

## DIFFERENTIATION-SUPPRESSIVE POLYPEPTIDE

### BACKGROUND OF THE INVENTION

This invention relates to a novel bioactive substance which suppresses differentiation of undifferentiated cells.

### DESCRIPTION OF THE RELATED ART

Human blood and lymph contain various types of cells and each cell plays important roles. For example, the erythrocyte carries oxygen; platelets have hemostatic action; and lymphocytes prevent infection. These various cells originate from hematopoietic stem cells in the bone marrow. Recently, it has been clarified that the hematopoietic stem cells are differentiated to various blood cells. Osteoclasts and mast cells by stimulation of various cytokines in vivo and environmental factors. In the cytokines, there have been found, for example, erythropoietin (EPO) for differentiation to erythrocytes; granulocyte colony stimulating factor (G-CSF) for differentiation to leukocytes; and platelet growth factor (mpl ligand) for differentiation to megakaryocytes which are platelet producing cells, and the former two have already been clinically applied.

The undifferentiated blood cells are generally classified into two groups consisting of blood precursor cells which are destined to differentiate to specific blood series and hematopoietic stem cells which have differentiation ability to all series and self-replication activity. The blood precursor cells can be identified by various colony assays. However, identification method for the hematopoietic stem cells have not been established. In these cells, stem cell factor (SCF), interleukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-6 (IL-6), interleukin-1 (IL-1) granulocyte colony

stimulating factor (G-CSF) and oncostatin M have been reported to stimulate cell differentiation and proliferation. Trials for expansion of hematopoietic stem cells in vitro have been examined in order to replace bone marrow transplantation for applying hematopoietic stem cell transplantation therapy or gene therapy. However, when the hematopoietic stem cells are cultured in the presence of the above mentioned cytokines, multi-differentiation activities and self-replication activities, which are originally in the position of the hematopoietic stem cells, gradually disappeared and are changed to the blood cell precursors which are only to differentiate to specific series after 5 weeks of cultivation, and multi-differentiation activity which is one of the specific features of the hematopoietic stem cells, is lost (Wagner et al., Blood 86. 512-523, 1995).

For proliferation of the blood precursor cells, a single cytokine is not sufficient, but the synergistic action of several cytokines are important. Consequently, in order to proliferate the hematopoietic stem cells while maintaining specific features of the hematopoietic stem cells, it is necessary to add cytokines which suppress differentiation together with the cytokines which proliferate and differentiate the undifferentiated blood cells. In general, many cytokines which stimulate proliferation or differentiation of cells are known, but small numbers of cytokines which suppressed cell differentiation are known. For example, leukemia inhibitory factor (LIF) has an action of proliferation of mouse embryonic stem cells without differentiation, but it has no action against the hematopoietic stem cells or blood precursor cells. Transforming growth factor (TGF -  $\beta$ ) has suppressive action for proliferation against various cells, but no fixed actions against the hematopoietic stem cells or blood precursor cells.

Not only blood cells but also undifferentiated cells, especially stem cells, are thought to be involved in tissue regeneration. These regeneration of tissues and

proliferation of undifferentiated cells in each tissue can be applied in various ways by referring to the known reference (Katsutoshi Yoshizato, Regeneration - a mechanism of regeneration, 1996, Yodosha Publ. Co.).

Notch is a receptor type membrane protein which is involved in regulation of nerve cells differentiation found in Drosophila. Homologues of the Notch are found in various animal kinds exceeding to the invertebrate and vertebrate including nematoda (Lin-12). Xenopus laevis (Xotch), mouse (Motch) or human (TAN-2). Ligand of the Notch in Drosophila are known. These are Drosophila Delta (Delta) and Drosophila Serrate (Serrate). Notch ligand homologues are found in various animal kinds as similar to the Notch of receptors (Artavanis-Tsakonas et al., Science 268, 225-232, 1995).

Human Notch homologue, TAN-1 is found widely in the tissues in vivo (Ellisen et al., Cell 66. 649-661, 1991). Two Notch analogous molecules other than TAN-1 are reported (Artavanis-Tsakonas et al., Science 268, 225-232, 1995). Expression of TAN-1 was also observed in CD34 positive cells in blood cells by PCR (Polymerase Chain Reaction) (Milner et al., Blood 83, 2057-2062, 1994). However, in relation to humans, gene cloning of human Delta and human Serrate, which are thought to be the Notch ligand, have not been reported.

In Drosophila Notch, binding with the ligand was studied and investigated in detail, and it was found that the Notch can be bound to the ligand with  $Ca^{++}$  at the binding region, which is a repeated amino acid sequence No. 11 and No. 12 in the amino acid sequence repeat of Epidemal Growth Factor (EGF) like repeating (Fehon et al., Cell 61. 523-534, 1990, Rebay et al., ibid. 67, 687-699, 1991 and Japan. Patent PCT Unexam. Publ. 7-503123). EGF-like repeated sequences are conserved in Notch homologues of the other species. Consequently, the same mechanism in binding with ligand is estimated. An

amino acid sequence which is called DSL (Delta-Serrate-Lag-2) near the amino acid terminal, and EGF-like repeated sequence as in the receptor are conserved in the ligand (Artavanis-Tsakonas et al.), Science 268, 225-232, 1995).

The sequence of DSL domain is not found except for the Notch ligand molecules, and is specific to Notch ligand molecule. A common sequence of DSL domain is shown in the sequence listing, SEQ ID NO: 1 in general formula, and comparison with human Delta-1 and human Serrate-1 of the present invention and known Notch ligand molecules are shown in Fig. 1.

EGF-like sequence has been found in thrombomodulin (Jackman et al., Proc. Natl. Acad. Sci. USA 83, 8834-8838, 1986), low density lipoprotein (LDL) receptor (Russell et al., Cell 37, 577-585, 1984), and blood coagulating factor (Furie et al., Cell 53, 505-518, 1988), and is thought to play important roles in extracellular coagulation and adhesion.

Recently, the vertebrate homologues of the cloned Drosophila Delta were found in chicken (C-Delta-1) and Xenopus laevis (X-Delta-1), and it was reported that X-Delta-1 had acted through Notch in the generation of the protoneuron (Henrique et al., Nature 375, 787-790, 1995 and Chitnis et al., ibid. 375, 761-766, 1995). Vertebrate homologue of Drosophila Serrate was found in rat as rat Jagged (Jagged) (Lindsell et al., Cell 80, 909-917, 1995). According to the Lindsell et al., mRNA of the rat Jagged is detected in the spinal cord of fetal rats. As a result of cocultivation of a myoblast cell line is found. However, the rat Jagged has no action against the myoblast cell line without forced expression of the rat Notch.

Considering the above reports, the Notch and ligand thereof may be involved in the differentiation regulation of the nerve cells, however, except for some myoblast cells,

their actions against cells including blood cells, especially primary cells, are unknown.

In the Notch ligand molecule, from the viewpoint of the prior studies on Drosophila and nematodae, the Notch ligand has specifically a structure of DSL domain which is not found other than in the Notch ligand. Consequently, the fact of having DSL domain means equivalent to ligand molecule for the Notch receptor.

### SUMMARY AND OBJECTS OF THE INVENTION

As mentioned above, concerning undifferentiated cells, proliferation that maintains their specificities has not been achieved. Major reasons are that factors suppressing differentiation of the undifferentiated cells are not sufficiently known. An object of the present invention is to provide a compound originated from novel factors which can suppress differentiation of the undifferentiated cells.

We have set up a hypothesis that the Notch and its ligand have action of differential regulation not only for neuroblasts and myoblasts but also for various undifferentiated cells, especially blood undifferentiated cells. However, in case of clinical application in the humans, prior known different species such as chicken or *Xenopus laevis* type notch ligand have problems of species specificities and antigenicities. Consequently, to obtain previously unknown human Notch ligand is essentially required. We had an idea that a molecule having DSL domain and EGF-like domain which are common to Notch ligand molecules and a ligand of the human Notch (TAN-1 etc.), which is a human Delta Homologue (hereinafter designated as human Delta) and human Serrate homologue (hereinafter designated as human Serrate), may be found. Also we have an idea that these findings may be a candidate for drugs useful for differential regulation of the undifferentiated cells, and we have tried to find out the same.

In order to find out human Notch ligands, we have analyzed amino acid sequences which are conserved in animals other than humans, and tried to find out genes by PCR using mixed primers of the corresponding DNA sequence. As a result of extensive studies, we have succeeded in isolation of cDNAs coding amino acid sequences of two new molecules, novel human Delta-1 and novel human Serrate-1, and have prepared the expression systems of protein having various forms using these cDNAs. Also we have established purification method of the proteins which were purified and isolated.

Amino acid sequences of novel human Delta-1 are shown in the sequence listings, SEQ ID NO: 2-4. DNA sequence coding these sequence is shown in the sequence listing, SEQ ID NO: 8. Amino acid sequence of novel human Serrate-1 is shown in the sequence listings, SEQ ID NO: 5-7. DNA sequence coding these sequence is shown in the sequence listing. SEQ ID NO: 10.

Physiological actions of the these prepared proteins were searched by using nerve undifferentiated cells, preadipocytes, hepatocytes, myoblasts, skin undifferentiated cells, blood undifferentiated cells and immuno undifferentiated cells. As a result, we have found that novel human Delta-1 and novel human Serrate-1 had an action of differentiation-suppressive action to primary blood undifferentiated cells, and had a physiological action to maintain undifferentiated state.

Such actions to the blood undifferentiated cells have never been reported previously, and is a new discovery. No significant toxic actions were noted in the toxicity studies on mice, and useful pharmaceutical effects were suggested. Consequently, the pharmaceutical preparations containing the molecule of the present invention, medium containing the molecule of the present invention, and the device having immobilized thereon the molecule of the present invention are novel drugs and medical materials which

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sequence of SEQ ID NO: 4 of the sequence listing, the DNA having DNA sequence 242-2347 of the SEQ ID NO: 8 of the sequence listing, the DNA coding the polypeptide having amino acid sequence of SEQ ID NO: 6 of the sequence listing, the DNA having DNA sequence 502-3609 of SEQ ID NO: 10 of the sequence listing, the DNA coding the polypeptide having amino acid sequence of SEQ ID NO: 7 of the sequence listing, and the DNA having DNA sequence 502-4062 of SEQ ID NO: 10 of the sequence listing. The present invention still further relates to a recombinant DNA made by ligating a DNA selected from the groups of DNA hereinabove and a vector DNA which can express in the host cell, a cell transformed by the recombinant DNA, and a process for production of polypeptide by culturing cells and isolating the thus produced compound. The present invention still further relates to an antibody specifically recognizing the polypeptide having the amino acid sequence of SEQ ID NO: 7 of the sequence listing.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is explained in details in the following:

Preparation of cDNA necessary for gene manipulation, expression analysis by Northern blotting, screening by hybridization, preparation of recombinant DNA, determination of DNA base sequence and preparation of cDNA library, all of which are series of molecular biological experiments, can be performed according to a description of the conventional textbook for the experiments. The above conventional textbook of the experiments is, for example, Maniatis et al. ed. Molecular Cloning, A laboratory manual, 1989, Eds., Sambrook, J., Fritsch, E.F. and Maniatis, T., Cold Spring Harbor Laboratory Press.

A polypeptide of the present invention has at least one of the polypeptides in



the sequence listing SEQ ID NO: 1-7. A mutant and allele which naturally occur in the nature are included in the polypeptide of the present invention unless the polypeptides of the sequence listing, SEQ ID NO: 1-7 lose their properties. Modification and substitution of amino acids are described in details in the patent application by the name of Benntt et al. (National Unexam. Publ. WO96/2645) and can be prepared according to the description thereof.

A DNA sequence coding polypeptides of the sequence listing, SEQ ID NO: 2-4 is shown in the sequence listing, SEQ ID NO: 8, and a DNA sequence coding polypeptides of the sequence listing, SEQ ID NO: 5-7 is show in the sequence listing, SEQ ID NO: 10, together with their amino acid sequences. In these DNA sequences, even if amino acid level mutation is not generated, naturally isolated chromosomal DNA or cDNA thereof may have a possibility to mutate in the DNA base sequence as a result of degeneracy of genetic code without changing amino acid sequence coded by the DNA. A 5'-untranslated region and 3'-untranslated region are not involved in amino acid sequence determination of the polypeptide, so DNA sequences of these regions are easily mutated. The base sequence obtained by these degeneracies of genetic codes is included in the DNA of the present invention.

Undifferentiated cells in the present invention are defined as cells which can grow by specific stimulation, and cells which can be differentiated to the cells having specific functions as a result of the specific stimulations. These include undifferentiated cells of the skin tissues, undifferentiated cells of the brain and nervous systems, undifferentiated cells of the muscular systems and undifferentiated cells of the blood cells. These cells include the cells of self-replication activity which are called stem cells, and the cells having an ability to generate the cells of these lines. The differentiation-suppressive

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action means suppressive action for autonomous or heteronomous differentiation of the undifferentiated cells, and is an action for maintaining undifferentiated condition. The brain and nervous undifferentiated cells can be defined as cells having ability to differentiate to the cells of the brain or nerve having specific functions by specific stimulation. The undifferentiated cells of the muscular systems can be defined as cells having ability to differentiate to the muscular cells having specific functions by specific stimulation. The blood undifferentiated cells in the present invention can be defined as cell groups consisting of the blood precursor cells which are differentiated to the specific blood series identified by blood colony assay, and hematopoietic stem cells having differentiation to every series and self-replication activities.

In the sequence listing, amino acid sequence in SEQ ID NO: 1 shows general formula of common amino acid sequence of DSL domain which is a common domain structure of the Notch ligand molecules, and at least this domain structure corresponds to the sequence listing, AMINO ACIDS 158-200 of the human Delta-1, or the sequence listing AMINO ACIDS 156-198 of the human Serrate-1.

The amino acid sequence in the sequence listing, SEQ ID NO: 2 is a sequence of the active center of the present invention of human Delta-1 minus the signal peptide, i.e. amino acid sequence from the amino terminal to DSL domain, and corresponds to an amino acid No. 1 to 200 in SEQ ID NO: 4 of the mature full length amino acid sequence of human Delta-1 of the present invention. The amino acid sequence in SEQ ID NO: 3 is amino acid sequence of extracellular domain of the present invention of human Delta-1 minus the signal peptide, and corresponds to an amino acid No. 1 to 520 in SEQ ID NO: 4 of the mature full length amino acid sequence of human Delta-1 of the present invention. The amino acid sequence of SEQ ID NO: 4 is the mature full length amino acid sequence

of the human Delta-1 of the present invention.

The amino acid sequence in the sequence listing, SEQ ID NO: 5 is a sequence of the active center of the present invention of human Serrate-1 minus the signal peptide, i.e. amino acid sequence from the amino terminal to DSL domain, and corresponds to an amino acid No. 1 to 198 in SEQ ID NO: 7 of the mature full length amino acid sequence of human Serrate-1 of the present invention. The amino acid sequence in SEQ ID NO: 6 is amino acid sequence of extracellular domain of the present invention of human Serrate-1 minus the signal peptide, and corresponds to an amino acid No. 1 to 1036 in SEQ ID NO: 7 of the mature full length amino acid sequence of human Serrate-1 of the present invention. The amino acid sequence of SEQ ID NO: 7 is the mature full length amino acid sequence of the human Serrate-1 of the present invention.

The sequence of SEQ ID NO: 8 is the total amino acid sequence of human Delta-1 of the present invention and cDNA coding the same, and the sequence of SEQ ID NO: 10 is total amino acid sequence of human Serrate-1 of the present invention and cDNA coding the same.

The left and right ends of the amino acid sequences in the sequence listing indicate amino terminal (hereinafter designated as N-terminal) and carboxyl terminal (hereinafter designated as C-terminal), respectively, and the left and right ends of the nucleotide sequences are 5'-terminal and 3'-terminal, respectively.

Cloning of human Notch ligand gene can be performed by the following method. During the evolution of the organisms, a part of amino acids sequences of the human Notch ligand is conserved. DNA sequence corresponding to the conserved amino acid sequence is designed, and is used as a primer of RT-PCR (Reverse Transcription Polymerase Chain reaction), then a PCR template of the human origin is amplified by PCR

reaction, thereby fragments of human Notch ligand can be obtainable. Furthermore, RT-PCR primer is prepared by applying the known DNA sequence information of the Notch ligand homologue of the organisms other than humans, and the known gene fragments can be possibly obtained from PCR template of the said organisms.

In order to perform PCR for obtaining fragments of human Notch ligand, PCR for DSL sequence is considered, but a large number of combinations of DNA sequence corresponding to amino acid sequence conserved in this region can be expected, and a design for PCR is difficult. As a result, PCR of the EGF-like sequence has to be selected. As explained hereinbefore, since EFG-like sequence is conserved in a large number of molecules, to obtain the fragments and identification are extremely difficult.

We have designed and prepared about 50 PCR primer sets, for example, the primer set of the sequence shown in Example 1, PCR was performed with these primer sets by using PCR template of cDNA prepared from poly A+ RNA of various tissues of human origin, and more than 10 PCR products from each tissue were subcloned, as well as performing sequencing for more than 500 types. A clone having a desired sequence could be identified. Namely, the obtained PCR product is cloned in the cloning vector, transforming the host cells by using recombinant plasmid which contains the PCR product, culturing the host cells containing the recombinant plasmid on a large scale, purifying and isolating the recombinant plasmid, checking the DNA sequence of PCR product which is inserted into the cloning vector, and trying to obtain the gene fragment which may have a sequence of human Delta-1 by comparing with the sequence of the known Delta of other species. We have succeeded to find out the gene fragment which contains a part of cDNA of human Delta-1, the same sequence of DNA sequence from 1012 to 1375 described in the sequence listing, SEQ ID NO: 8.

We have also designed and prepared about 50 PCR primer sets, for example, the primer set of the sequence shown in Example 3, and PCR was performed with these primer sets by using PCR template of cDNA prepared from poly A+ RNA of various tissues of human origin, and more than 10 PCR products from each tissue were subcloned, as well as performing sequencing for more than 500 types. A clone having a desired sequence could be identified. Namely, the obtained PCR product is cloned in the cloning vector, transforming the host cells by using recombinant plasmid which contains the PCR product, culturing the host cells containing the recombinant plasmid on a large scale, purifying and isolating the recombinant plasmid, checking the DNA sequence of PCR product which is inserted into the cloning vector, and trying to obtain the gene fragment which may have a sequence of human Serrate-1 by comparing with the sequence of the known Serrate of other species. We have succeeded to find out the gene fragment which contains a part of cDNA of human Serrate-1, the same sequence of DNA sequence from 1272 to 1737 described in the sequence listing, SEQ ID NO: 10.

A full length of the objective gene can be obtained from the human genomic gene library or cDNA library by using the thus obtained human Delta-1 fragment or human Serrate-1 gene fragment. The full length cloning can be made by isotope labeling and non-isotope labeling with the partial cloning gene, and screening the library by hybridization or other method. Isotope labeling can be performed by, for example, terminal labeling by using [ $^{32}\text{P}$ ]  $\gamma$ -ATP and T4 polynucleotide kinase, or other labeling methods such as nick translation or primer extension method can be applied. In another method, human originated cDNA library is ligated into the expression vector, expressing by COS-7 or other cells, and screening the objective gene by expression cloning to isolate cDNA of the ligand. In the expression cloning, a cell sorter fractionation method which is applied with

binding with polypeptide containing amino acid sequence of prior known 4 Notches such as TAN-1, and a detection method by film emulsion using radioisotope can be mentioned. In this specification, methods for obtaining genes of human Delta-1 and human Serrate-1 are explained, and in addition to that obtaining the Notch ligand homologue gene of the other organism is important for analysis of ligand action. This may be made by the same treatment. The obtained gene is subjected to DNA sequence determination and amino acid sequence can be estimated.

As shown in Example 2, gene fragments containing human Delta-1 PCR product are labeled with radioisotope to prepare hybridization probe screening is performed using cDNA of human placenta origin as the screening library. DNA sequences of the thus obtained clones are determined, and the clone is obtained containing DNA nucleotide sequence shown in the sequence listing. SEQ ID NO: 8, and shown to have the amino acid sequence coded in the sequence listing. SEQ ID NO: 4. We have succeeded in cloning cDNA coding full length of amino acids sequence of human Delta-1.

These sequences were compared with the data base (Genbank release 89, June, 1995), and found that these were novel sequences. The said amino acid sequence was analyzed in hydrophilic part and hydrophobic part according to a method by Kyte-Doolittle (J. Mol. Biol. 157: 105, 1982). A result indicated that human Delta-1 of the present invention is expressed on cells as a cellular membrane protein having a transmembrane domain.

As shown in Example 4, gene fragments containing human Serrate-1 PCR product are labeled with radioisotope to prepare hybridization probe, screening is performed using cDNA of human placenta origin as the screening library, DNA sequences of the thus obtained clones are determined and the clone is obtained containing DNA

nucleotide sequence shown in the sequence listing, SEQ ID NO: 10, and shown to have the amino acid sequence coded in the sequence listing, SEQ ID NO: 7. In this screening, an intracellular part of gene sequence coding a full length of amino acids sequence, namely a peripheral part of termination codon cannot be cloned. Consequently, as shown in Example 4, gene cloning is performed by RACE method (rapid amplification of cDNA ends, Frohman et al., Proc. Natl. Acad. Sci. U.S.A. 85, 8998-9002, 1988) and finally succeeded in cloning of cDNA coding full length of amino acid sequence of human Serrate-1.

These sequences were compared with the data base (Genbank release 89, June, 1995, and found that these were novel sequences. The said amino acid sequence was analyzed in hydrophilic part and hydrophobic part according to a method by Kyte-Doolittle (J. Mol. Biol. 157: 105, 1982). A result indicated that human Serrate-1 of the present invention is expressed on cells as a cellular membrane protein having a transmembrane domain.

Examples of plasmids integrated with cDNA are, for example, E. coli originated pBR322, pUC18, pUC19, pUC118 and pUC119 (Takara Shuzo Co., Japan), but other plasmids can be used if they can replicate and proliferate in the host cells. Examples of phage vectors integrated with cDNA are, for example,  $\lambda$ gt10 and  $\lambda$ gt11, but other vectors can be used if they can grow in the host cells. The thus obtained plasmids are transduced into suitable host cells such as genus Escherichia and genus Bacillus using calcium chloride method. Examples of the above genus Escherichia are Escherichia coli K12HB101, MC1061, LE392 and HM109. Example of the above genus Bacillus is Bacillus subtilis MI114. Phage vector can be introduced into the proliferated E. coli by the in vitro packaging method (Enquist and Sternberg, Meth. Enzymol., 68, 281-, 1979).

According to the analysis of amino acid sequence of the human Delta-1, amino acid sequence of a precursor of human Delta-1 consists of 723 amino acids residue shown in the sequence listing, SEQ ID NO: 8, and the signal peptide domain is estimated to correspond to an amino acid sequence of 21 amino acids residue from No. -21 methionine to No. -1 serine of the sequence listing; extracellular domain: 520 amino acids residue from No. 521 proline to No. 552 leucine; and intracellular domain: 150 amino acids region from No. 553 glutamine to No. 702 valine. These domains are estimated domain construction from amino acid sequences, and actual presence form may differ from the above structure, and constitutional amino acids of each domain hereinabove defined may have possibility to change 5 to 10 amino acids sequence.

According to a comparison in amino acid sequence of human Delta-1 and Delta homologue of the other organisms, the homologies with *Drosophila* Delta, chick Delta and *Xenopus laevis* are 47.6%, 83.3% and 76.2%, respectively. The human Delta-1 of the present invention is different from these Deltas and is novel substance which is clarified at first by the present inventors. Search from all of organisms in the above data base indicated that polypeptides having the identical sequence of the human Delta-1 could not be found.

The homologues of Notch ligand have evolutionally conserved common sequence, i.e. repeated DSL sequence and EGF-like sequence. As a result of comparison with amino acid sequence of human Delta-1, these conserved sequence is estimated. Namely, DSL sequence corresponds to 43 amino acids residue from No. 158 cysteine to No. 200 cysteine of the amino acid sequence in the sequence listing, SEQ ID NO: 4. EGF-like sequence exists with 8 repeats wherein, in the amino acid sequence in the sequence listing SEQ ID NO: 4, the first EGF-like sequence from No. 205 cysteine to No. 233



cysteine; the second EGF-like sequence from No. 236 cysteine to No. 264 cysteine; the third EGF-like sequence from No. 271 cysteine to No. 304 cysteine; the fourth EGF-like sequence from No. 311 cysteine to No. 342 cysteine; the fifth EGF-like sequence from No. 349 cysteine to No. 381 cysteine; the sixth EGF-like sequence from No. 388 cysteine to No. 419 cysteine; the seventh EGF-like sequence from No. 426 cysteine to No. 457 cysteine; and the eighth EGF-like sequence from No. 464 cysteine to No. 495 cysteine.

A part of sugar chain attached is estimated from amino acid sequence of the human Delta-1 may be No. 456 asparagine residue in the sequence listing, SEQ ID NO: 4 as a possible binding site of N-glycoside bonding for N-acetyl-D-glucosamine. O-glycoside bond of N-acetyl-D-galactosamine is estimated to be a serine or threonine residue rich part. Protein bound with sugar chain is generally thought to be stable in vivo and to have strong physiological activity. Consequently, in the amino acid sequence of polypeptide having sequence of the sequence listing, SEQ ID NO: 2, 3 or 4, polypeptides having N-glucoside or O-glucoside bond with sugar chain of N-acetyl-D-glucosamine or N-acetyl-D-galactosamine is included in the present invention.

According to the analysis of amino acid sequence of the human Serrate-1, amino acid sequence of a precursor of human Serrate-1 consists of 1218 amino acids residue shown in the sequence listing, SEQ ID NO: 10, and the signal peptide domain is estimated to correspond 31 amino acids residue in the amino acid sequence from No. -31 methionine to No. -1 alanine of the sequence listing; extracellular domain: 1036 amino acids residue from No. 1 serine to No. 1036 asparagine; transmembrane domain: 26 amino acids residue from No. 1037 phenylalanine to No. 1062 leucine; and intracellular domain: 106 amino acids domain from No. 1063 arginine to No. 1187 valine. These domains are estimated domain construction from amino acid sequences, and actual presence form may

differ from the above structure, and constitutional amino acids of each domain hereinabove defined may have possibility to change 5 to 10 amino acids sequence.

According to a comparison in amino acid sequence of human serrate-1 and Serrate homologue of the other organisms, the homologies with Drosophila Serrate, and rat Jagged are 32.1% and 95.3%, respectively. The human Serrate-1 of the present invention is different from these Serrates and is novel substance which is clarified at first by the present inventors. Search from all of organisms in the above data base indicated that polypeptides having the identical sequence of the human Serrate-1 could not find out.

The homologues of Notch ligand have evolutionally conserved common sequence, i.e. repeated DSL sequence and EGF-like sequence. As a result of comparison with amino acid sequence of human Serrate-1 and other Notch ligand homologues, these conserved sequence is estimated. Namely, DSL sequence corresponds to 43 amino acids residue from No. 156 cysteine to No. 198 cysteine of the amino acid sequence in the sequence listing, SEQ ID NO: 7. EGF-like sequence exists with 16 repeats wherein, in the amino acid sequence in the sequence listing, SEQ ID NO: 7, the first EGF-like sequence from No. 205 cysteine to No. 231 cysteine; the second EGF-like sequence from No. 234 cysteine to No. 262 cysteine; the third EGF-like sequence from No. 269 cysteine to No. 302 cysteine; the fourth EGF-like sequence from No. 309 cysteine to No. 340 cysteine; the fifth EGF-like sequence from No. 356 cysteine to No. 378 cysteine; the sixth EGF-like sequence from No. 423 cysteine to No. 453 cysteine; the eighth EGF-like sequence from No. 462 cysteine to No. 453 cysteine; the ninth EGF-like sequence from No. 498 cysteine to No. 529 cysteine; the 10<sup>th</sup> EGF-like sequence from No. 536 cysteine to No. 595 cysteine; the 11<sup>th</sup> EGF-like sequence from No. 602 cysteine to No. 633 cysteine; the 12<sup>th</sup> EGF-like sequence from No. 640 cysteine to No. 671 cysteine; the 13<sup>th</sup> EGF-like sequence

from No. 678 cysteine to No. 709 cysteine; the 14<sup>th</sup> EGF-like sequence from No. 717 cysteine to No. 748 cysteine; and the 16<sup>th</sup> EGF-like sequence from No. 793 cysteine to No. 824 cysteine. However, the 10<sup>th</sup> EGF-like sequence has irregular sequence containing 10 residues of cysteine.

A part of sugar chain attached is estimated from amino acid Sequence of the human Serrate-1 may be No. 112, 131, 186, 351, 528, 554, 714, 1014 and 1033 asparagine residue in the sequence listing, SEQ ID NO: 7 as a possible binding site of N-glycoside bonding for N-acetyl-D-glycosamine. O-glycoside bond of N-acetyl-D-galactosamine is estimated to be a serine or threonine residue rich part, Protein bound with sugar chain is generally thought to be stable in vivo and to have strong physiological activity. Consequently, in the amino acid sequence of polypeptide having sequence of the sequence listing, SEQ ID NO: 5, 6 or 7, polypeptides having N-glucoside or O-glucoside bond with sugar chain of N-acetyl-D-glucosamine or N-acetyl-D-galactosamine is included in the present invention.

As a result of studies on binding of Drosophila Notch and its ligand, amino acid region necessary for binding with ligand of Drosophila Notch with the Notch is from N-terminal to DSL sequence of the matured protein, in which signal peptide is removed (Japan. Pat. PCT Unexam. Publ. No. 7-503121). This fact indicates that a domain necessary for expression of ligand action of human Notch ligand molecule is at least the DSL domain, i.e. a domain containing amino acid sequence of the sequence listing, SEQ ID NO: 1, and a domain at least necessary for expression of ligand action of human Delta-1 is novel amino acid sequence shown in the sequence listing, SEQ ID NO: 2, and further a domain at least necessary for expression of ligand action of human Serrate-1 is novel amino acid sequence shown in the sequence listing, SEQ ID NO: 5.

An mRNA of human Delta-1 can be detected by using DNA coding a part or all of gene sequence in the sequence listing, SEQ ID NO: 8, and an mRNA of human Serrate-1 can be detected by using DNA coding a part or all of gene sequence in the sequence listing, SEQ ID NO: 10. For example, a method for detection of expression of these genes can be achieved by applying with hybridization or PCR by using complementary nucleic acids of above 12mer or above 16mer, preferably above 18mer having nucleic acid sequence of a part of sequence in the sequence listing, SEQ ID NO: 8 or 10, i.e. antisense DNA or antisense RNA, its methylated, methylphosphated, deaminated, or thiophosphated

derivatives. By the same method, detection of homologues of the gene of other organisms such as mice or gene cloning can be achieved. Further cloning of genes in the genome including humans can be made. Using these genes cloned by such like methods, further detailed functions of the human Delta-1 or human Serrate-1 of the present invention can be clarified. For example, using the modern gene manipulation techniques, every methods including transgenic mouse, gene targeting mouse or double knockout mouse in which genes relating to the gene of the present invention are inactivated, can be applied. If abnormalities in the genome of the present gene is found, application to gene diagnosis and gene therapy can be made.

A transformant in which vector pUCDL-1F, which contains cDNA coding total amino acid sequence of human Delta-1 of the present invention, is transformed into E.coli JM109, has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, MITI, of 1-1-3, Higasi, Tsukuba-shi, Ibaragi-ken, Japan, as E. coli : JM109-pUCDL-1F. Date of deposit was October 28, 1996, and deposition No. is FBRM BP-5728. A transformant in which vector pUCSR-1, which contains cDNA coding total amino acid sequence of human Serrate -1 of the present invention, is transformed into E.coli JM109, has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, MITI, of 1-1-3, Higasi, Tsukuba-shi, Ibaragi-ken, Japan, as E. coli : JM109-pUCSR-1. Date of deposit was October 28, 1996, and deposition No. is FBRM BP-5726.

Exprssion and purification of various forms of human Delta-1 and human Serrate-1 using cDNA coding amino acid sequence of human Delta-1 and human Serrate-1 isolated by the above methods are known in the references (Kriegler, Gene Transfer and Expression- A Laboratory Manual Stockton Press, 1990 and Yokota et al. Biomanual Series 4, Gene transfer and expression and analysis, Yodosha Co., 1994). A cDNA coding the amino acid sequence of the isolated said human Delta-1 and human Serrate-1 is ligated to preferable expression

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T06211 26556660

vector and is produced in the host cells of eukaryotic cells such as animal cells and insect cells or prokaryotic cells such as bacteria.

In the expression of human Delta-1 and human Serrate-1 of the present invention, DNA coding polypeptide of the present invention may have the translation initiation codon in 5'-terminal and translation termination codon in 3'-terminal. These translation initiation codon and translation termination codon can be added by using preferable synthetic DNA adapter. Further for expression of the said DNA, promoter is linkaged in the upstream of the DNA sequence. Examples of vector are plasmid originated from Bacillus, plasmid originated from yeast or bacteriophage such as  $\lambda$ -phage and animal virus such as retrovirus and vaccinia virus.

Examples of promoters used in the present invention are any promoters preferable for corresponding to the host cells used in gene expression.

In case that the host cell in the transformation is genus E. coli, lac-promoter, trp-promoter and lac-promoter are preferable, and in case of host of genus Bacillus, SP01 promoter and SP02 promoter are preferable, and in case of host of yeast, PGK promoter, GAP promoter and ADH promoter are preferable.

In case that the host cell is animal cells, a promoter originated from SV40 such as SR $\alpha$  promoter as described in Example 5, promoter of retrovirus, metallothionein promoter and heatshock promoter can be applied.

Polypeptide of the present invention can be expressed by using the expression vector having ability to be used by any person skilled in the arts.

Expression of the polypeptide of the present invention can be made by using only DNA coding the amino acid sequence of the sequence listing, SEQ ID NO: 2, 3, 4, 5, 6 or 7. However, the protein added with specific function can be produced by using DNA, to which added cDNA coding the known antigen epitope for easier detection of the produced polypeptide or added cDNA coding the immunoglobulin Fc for forming multimer.

As shown in Example 5, we have prepared expression vectors, which express extracellular proteins of human Delta-1, as follows:

- 1) DNA coding the amino acids from No. 1 to 520 in amino acid sequence in the sequence listing, SEQ ID NO: 3,
- 2) DNA coding chimera protein to which added polypeptide having 8 amino acid, i.e. an amino acid sequence consisting of Asp Tyr Lys Asp Asp Asp Asp Lys (hereinafter designates FLAG sequence, the sequence listing, SEQ ID NO: 12), in the C-terminal of the amino acids from No. 1 to 520 in amino acid sequence in the sequence

listing, SEQ ID NO: 3, and

3) DNA coding chimera protein to which added Fc sequence below the hinge region of human IgG1 (refer to International Patent Publ. WO96/11221 in the C-terminal of the amino acids from No. 1 to 520 in amino acid sequence in the sequence listing, SEQ ID NO:3, and to have dimer structure by disulfide bond in the hinge region, are ligated individually with the expression vector pMKITNeo (Maruyama et al. Japan Molecular Biology Soc. Meeting Preliminary lecture record, obtainable from Dr. Maruyama in Tokyo Medical and Dental College, containing promoter SR $\alpha$ ) to prepare extracellular expression vectors of human Delta-1.

The full-length expression vectors of the human Delta-1 as the expression vectors, which express full-length proteins of the human Delta-1, can be prepared as follows.

4) DNA coding amino acids from No. 1 to 702 in the sequence listing, SEQ ID NO: 4 and

5) DNA coding chimera protein to which added polypeptide having FLAG sequence in the C-terminal of amino acids from No. 1 to 702 in the sequence listing, SEQ ID NO: 4

are ligated individually with the expression vector pMKITNeo to prepare the full-length expression vectors of human Delta-1. The transformant is prepared by using expression plasmid containing DNA coding the thus constructed said human Delta-1.

As shown in Example 6, we have prepared expression vectors, which express extracellular proteins of human Serrate-1, as follows.

6) DNA coding the amino acids from No. 1 to 1036 in amino acid sequence in the sequence listing, SEQ ID NO: 6,

7) DNA coding chimera protein to which added polypeptide having FLAG sequence in the C-terminal of the amino acids from No. 1 to 1036 in amino acid sequence in the sequence listing, SEQ ID NO: 6, and

8) DNA coding chimera protein to which added said Fc sequence in the C-terminal of the amino acids from No. 1 to 1036 in amino acid sequence in the sequence listing, SEQ ID NO: 6, and to have dimer structure by disulfide bond in the hinge region.

are ligated individually with the expression vector pMKITNeo to prepare extracellular expression vectors of human Serrate-1.

The full-length expression vectors of the human Serrate-1 as the expression vectors, which express full-length proteins of the human Serrate-1, can be prepared as follows.

9) DNA coding amino acids from No. 1 to 1187 in the sequence listing, SEQ ID NO: 7 and

10) DNA coding chimera protein to which added polypeptide having FLAG sequence in the C-terminal of amino acids from No. 1 to 1187 in the sequence listing, SEQ ID NO: 7

are ligated individually with the expression vector pMKITNeo to prepare the full-length expression vectors of human Serrate-1. The transformant is prepared by using expression plasmid containing DNA coding the thus constructed said human Serrate-1.

Examples of the host are genus *Escherichia*, genus *Bacillus*, yeast and animal cells. Examples of animal cells are simian cell COS-7 and Vero, Chinese hamster cell CHO and silk worm cell SF9.

As shown in Example 7, the above expression vectors 1) - 10) are transduced individually; the human Delta-1 or human Serrate-1 are expressed in COS-7 cell (obtainable from the Institute of Physical and Chemical Research, Cell Development Bank, RCB0539), and the transformants which were transformed by these expression plasmids, can be obtained. Further, human Delta-1 polypeptide and human Serrate-1 polypeptide can be produced by culturing the transformants under preferable culture condition in medium by known culture method.

As shown in Example 8, human Delta-1 polypeptide and human Serrate-1 polypeptide can be isolated and purified from the above cultured mass, in general, by the following methods.

For extraction of the substance from cultured microbial cells or cells, microbial cells or cells are collected by known method such as centrifugation after the cultivation, suspended in preferable buffer solution, disrupted the microbial cells or cells by means of ultrasonication, lysozyme and/or freeze-thawing and collected crude extract by centrifugation or filtration. The buffer solution may contain protein-denaturing agents such as urea and guanidine hydrochloride or surface active agents such as Triton-X. In case of secretion in the cultured solution, the cultured mass is separated by the known method such as centrifugation to separate from microbial cells or cells and the supernatant solution is collected.

The thus obtained human Delta-1 or human Serrate-1, which are contained in the cell extracts or cell supernatants, can be purified by known protein purification methods. During the purification process, for confirmation of existence of the protein, in case of the fused proteins of the above FLAG and human IgGFc, they can be detected by immunoassay using antibody against

known antigen epitope and can be purified. In case of not to express as such the fused protein, the antibody in Example 9 can be used for detection.

Antibodies, which specifically recognize human Delta-1 and human Serrate-1, can be prepared as shown in Example 9. Antibodies can be prepared by the methods described in the reference (Antibodies a laboratory manual, E. Harlow et al., Cold Spring Harbor Laboratory) or recombinant antibodies expressed in cells by using immunoglobulin genes isolated by gene cloning method. The thus prepared antibodies can be used for purification of human Delta-1 and human Serrate-1. The human Delta-1 or human Serrate-1 can be detected and assayed by using antibodies which recognize specifically human Delta-1 or human Serrate-1 as shown in Example 9, and can be used for diagnostic agents for diseases accompanied with abnormal differentiation of cells such as malignant tumors.

More useful purification method is the affinity chromatography using antibody. Antibodies used in this case are antibodies described in Example 9. For fused protein, antibodies against FLAG in the case of FLAG, and protein G or protein A in the case of human IgGFc as shown in Example 8.

Any fused protein other than the protein as shown hereinabove can be used. For example, histidine Tag and myc-tag can be mentioned. Any fused proteins can be prepared by using methods of present day genetic engineering techniques other than the known methods, and peptides of the present invention derived from those fused proteins are in the scope of the present invention.

Physiological functions of the thus purified human Delta-1 and human Serrate-1 proteins can be identified by various assay methods, for example, physiological activity assaying methods using cell lines and animals such as mice and rats, assay methods of intracellular signal transduction based on molecular biological means, binding with Notch receptor etc.

We have observed actions for blood undifferentiated cells by using IgG1 chimera proteins of human Delta-1 and human Serrate-1.

As a result, we have found that, as shown in Example 10, in the umbilical cord blood derived blood undifferentiated cells, in which CD34 positive cell fraction is concentrated, polypeptides of the present invention have suppressive action of colony forming action against blood undifferentiated cells, which shows colony formation in the presence of cytokines. The suppressive action is only observed in the presence of SCF. This kind of effect has never been known.



As shown in Example 11, we have found that a maintenance of colony forming cells is significantly extended by addition of IgG1 chimera protein of human Delta-1 or human Serrate-1 in the long term (8 weeks) liquid culture in the presence of cytokines such as SCF, IL-3, IL-6, GM-CSF and Epo. Further we have found that the polypeptides of the present invention had an action not to suppress growth of the colony forming cells. A cytokine, MIP-1 $\alpha$  having migration and differentiation suppressive action of blood cells (Verfaillie et al., J. Exp. Med. 179, 643-649, 1994), has no action for maintaining undifferentiation for blood undifferentiated cells.

Further as shown in Example 12, we have found that as a result of adding IgG1 chimera protein of human Delta-1 or human Serrate-1 to the liquid culture in the presence of cytokines, the human Delta-1 and human Serrate-1 had activities for significantly maintaining LTC-IC (Long-Term Culture-Initiating Cells) number, which is positioned most undifferentiated blood stem cells in the human blood undifferentiated cells.

These results indicate that the human Delta-1 and human Serrate-1 suppress differentiation of blood undifferentiated cells, and these actions spread from blood stem cells to colony forming cells. These physiological actions are essential for in vitro expansion of blood undifferentiated cells. Cells cultured in the medium containing human Delta-1 or human Serrate-1 are efficient in recovery of suppression of bone marrow after administration of antitumor agents, accordingly in vitro growth of hemopoietic stem cells may be possible if other conditions would be completed. Further pharmaceuticals containing the polypeptide of the present invention have action protection and release of the bone marrow suppressive action, which is observed in adverse effects of antitumor agents.

Suppressive action for differentiation of cells in the undifferentiated cells other than blood cells is expected and stimulating action for tissue regeneration can be expected.

In the pharmaceutical use, polypeptides of the present invention are lyophilized with adding preferable stabilizing agents such as human serum albumin, and is used in dissolved or suspended condition with distilled water for injection when it is in use. For example, preparation for injection or infusion at the concentration of 0.1-1000  $\mu$ g/ml may be provided. A mixture of the compound of the present invention 1 mg/ml and human serum albumin 1 mg/ml divided in a vial could maintain activity of the said compound for long term. For culturing and activating cells in vitro, lyophilized preparation or liquid preparation of the polypeptide of the present invention

are prepared and are added to the medium or immobilized in the vessel for culture. Toxicity of the polypeptide of the present invention was tested. Any polypeptide, 10 mg/kg was administered intraperitoneally in mice, but no death of mice was observed.

In vitro physiological activity of the polypeptide of the present invention can be evaluated by administering to disease model mice or its resembled disease rats or monkeys, and examining recovery of physical and physiological functions and abnormal findings. For example, in case of searching abnormality in relation to hemopoietic cells, bone marrow suppressive model mice are prepared by administering 5-FU series of antitumor agents, and bone marrow cell counts, peripheral blood cell counts and physiological functions are examined in the administered group or the non administered group of mice. Further, in case of searching in vitro cultivation and growth of hemopoietic undifferentiated cells including hemopoietic stem cells, the bone marrow cells of mice are cultured in the groups with or without addition of the compound of the present invention, and the cultured cells are transferred into the lethal dose irradiated mice. Result of recovery is observed with the indications of survival rate and variation of blood counts. These results can be extrapolated to the humans, and accordingly useful effective data for evaluation of the pharmacological activities of the compound of the present invention can be obtained.

Applications of the compound of the present invention for pharmaceuticals include diseases with abnormal differentiation of cells, for example leukemia and malignant tumors. These are cell therapy, which is performed by culturing human derived cells in vitro while maintaining their original functions or adding new functions, and a therapy, which is performed by regenerating without damaging the functions originally existing in the tissues by administering the compound of the present invention under the regeneration after tissue injury. Amount of administration may differ in the type of preparation and ranges from 10  $\mu$ g/kg to 10 mg/kg.

Further strong physiological activity can be achieved by expression of forming multimer of the polypeptide of the present invention.

As shown in Example 10, since the suppressive action of human Delta-1 and human Serrate-1 is stronger in the IgG chimera protein having dimer structure, a form of stronger physiological activity is preferably expressed in the form of multimer formation.

Human Delta-1 and human Serrate-1 having multimer structure can be produced by a method of expressing chimera protein with human IgG Fc region

as described in the example and expressing the multimer having disulfide bond with hinge region of the antibody, or a method expressing chimera protein, in which antibody recognition region is expressed in the C-terminal or N-terminal, and reacting with the polypeptide containing extracellular part of the thus said Delta-1 and Human Serrate-1 and/<sup>the</sup> antibody which recognize specifically the antibody recognition region in the C-terminal or N-terminal. In the other methods, a method, in which a fused protein expressed with only the hinge region of the antibody and the dimerized by disulfide bond, can be mentioned. The multimer of human Delta-1 and human Serrate-1 having higher specific activity than the dimer can be obtained. The said multimer is constructed by fused protein which is prepared for expressing the peptide in the C-terminal, N-terminal or other region. The protein is prepared in the form of forming disulfide bond without effecting in any activities of the other human Delta-1 or human Serrate-1. The multimer structure can also be expressed by arranging one or more peptide, which is selected from polypeptides containing amino acids sequence of the sequence listing, SEQ ID NO: 2, 3, 5 or 6, with genetic engineering method in series or in parallel. Other known methods for providing multimer structure having dimer or higher can be applied. Accordingly, the present invention includes any polypeptides containing amino acid sequences described in the sequence listing, SEQ ID NO: 2, 3, 5 or 6 in the form of dimer or higher more structure prepared by genetic engineering technique.

Further in the other method, multimerization method using chemical cross-linker can be mentioned. For example, dimethylsuberimidate dihydrochloride for cross-linking lysine residue, N-( $\gamma$ -maleimidebutyryloxy) succinimide for cross-linking thiol group of cysteine residue and glutaraldehyde for cross-linking between amino groups can be mentioned. The multimer with dimer or more can be synthesized by applying these cross-linking reactions. Accordingly, the present invention includes any polypeptides containing amino acid sequences described in the sequence listing, SEQ ID NO: 2, 3, 5 or 6 in the form of dimer or more structure prepared by chemical cross-linking agents.

In application of medical care in which cells are proliferated and activated in vitro and are returned to the body, human Delta-1 or human Serrate-1 of the form hereinabove can be added directly in the medium, but immobilization can also be made. Immobilization method includes applying amino group or carboxyl group in the peptide, using suitable spacers or the above mentioned cross-linkers, and the polypeptide can be covalently bound to the culture vessels. Accordingly, the present invention includes any

polypeptides containing amino acid sequences described in the sequence listing, SEQ ID NO: 2, 3, 5 or 6 in the form of existing on the solid surface.

Since the natural human Delta-1 and human Serrate-1 are cell membrane proteins, differentiation suppressive action in the Examples can be expressed by cocultivating with cells expressing these molecules and blood undifferentiated cells. Consequently, this invention includes cocultivation method with transformed cells by using DNA coding amino acid sequences in the sequence listing, SEQ ID NO: 2 - 7 and undifferentiated cells.

Expressed cell may be COS-7 cell as shown in Examples, but cells of human origin are preferable, and further expressed cells may be cell line or any of human in vivo blood cells and somatic cells. Consequently, the polypeptide can be expressed in vivo by integrated into vectors for gene therapy.

As shown in Example 10, FLAG chimera protein of human Delta-1 or human Serrate-1, both of which are low concentrated monomer, shows not a colony formation suppressive action but a colony formation stimulating action. This action may be involved in expressing Notch receptor and Notch ligand in the occasion of cell division of blood undifferentiated cells and acting the polypeptide of the present invention as an antagonist for that action. This suggests that the polypeptide having amino acid sequence of the sequence listing, SEQ ID NO: 1, 2, 4 or 5, shows colony formation stimulation action by controlling the concentration of its action.

This fact suggests that inhibition of binding the polypeptide having amino acid sequence in the sequence listing, SEQ ID NO: 2 - 7 and these receptors can be used for finding out molecules and compounds for stimulating cell differentiation. The methods include binding experiment using radio isotope, luciferase assay using transcriptional control factors, a downstream molecule of the Notch receptor, and simulation on the computer by X-ray structural analysis. Accordingly, the present invention includes screening method for pharmaceuticals using polypeptide in the sequence listing, SEQ ID NO: 2 - 7.

As shown in Example 13, specific leukemia cells can be differentiated by using IgG chimera protein of human Delta-1 or human Serrate-1. Consequently, the present invention can be applied for diagnostic reagents for leukemia or isolation of specific blood cells. This result indicates that human Delta-1 or human Serrate-1 molecule binds specifically with its receptor, a Notch receptor molecule. For example, expression of Notch receptor can be detected by using fused protein with the above extracellular region and human IgGFc.

Notch is known to involve in some type of leukemia (Ellisen et al., Cell 66, 649-661, 1991). Accordingly, the polypeptide having amino acids sequence in the sequence listing, SEQ ID NO: 2, 3, 5 and 6 can be used for diagnostic reagents for in vitro or in vivo.

#### Brief explanation of the drawings

Fig. 1: Alignment of DSL domain of Notch ligand identified in various organisms including the molecules of the present invention, wherein the consensus sequence is SEQ ID NO: 40, hDelta-1.DSL is SEQ ID NO: 41, dDelta.DSL is SEQ ID NO: 42, xDelta.DSL is SEQ ID NO: 43, cDelta-1.DSL is SEQ ID NO: 44, mDelta-1.DSL is SEQ ID NO: 45, hSerrate-1.DSL is SEQ ID NO: 46, dSerrate.DSL is SEQ ID NO: 47, and rJagged.DSL is SEQ ID NO: 48.

Figs. 2A and 2B : Suppression of colony formation of the blood undifferentiated cells using the molecules of the present invention.

Fig. 3: Concentration dependency of colony formation suppression of the blood undifferentiated cells using the molecules of the present invention.

Fig. 4: A graph showing calculation of LTC-1 after liquid culture using the molecules of the present invention.

Figs. 5A and 5B: Cells stained by the molecules of the present invention.

#### Examples

Following examples illustrate the embodiments of the present invention but are not construed as limiting these examples.

#### Example 1

Cloning of PCR products using human Delta-1 primer and determination of base sequence

A mixed primer corresponding to amino acid sequence conserved in C-Delta-1 and X-Delta-1, i. e. sense primer DLTS1 (sequence listing, SEQ ID NO: 14) and antisense primer DLTA2 (sequence listing, SEQ ID NO: 15), were used.

A synthetic oligonucleotide was prepared by using automatic DNA synthesizer with the principle of immobilized method. - The automatic DNA synthesizer used was 391PCR-MATE of Applied Biosystems Inc., U.S.A. Nucleotide carrier immobilized with 3'-nucleotide, solution and reagents are used according to the instructions by the same corporation. Oligonucleotide was isolated from the carrier after finishing the designated coupling reaction and treating the oligonucleotide carrier, from which protective group of 5'-terminal was removed, with concentrated liquid ammonia at room temperature for one hour. For removing the protective groups of nucleic acid and phosphoric acid, the reactant solution containing nucleic acid was allowed to stand in the concentrated ammonium solution in the sealed vial at 55°C for over 14

hours. Each oligonucleotide, from which the carrier and protective groups were removed, was purified by using OPC cartridge of the Applied Biosystems Inc., and detritylated by using 2 % trifluoroacetic acid. Primer was dissolved in deionized water to set final concentration of 100 pmol/ $\mu$ l after purification.

Amplification of these primers by PCR was performed as follows. Human fetal brain originated cDNA mixed solution (QUICK-Clone cDNA, CLONTECH Inc., U.S.A.) 1  $\mu$ l was used. 10  $\times$  buffer solution [500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM MgCl<sub>2</sub>, 0.01 % gelatin] 5  $\mu$ l, dNTP mixture (Takara Shuzo Co., Japan) 4  $\mu$ l, sense primer DLTS1 (100 pmol/ $\mu$ l) 5  $\mu$ l which was specific to the above vertebrates and antisense primer DLTA2 (100 pmol/ $\mu$ l) 5  $\mu$ l and TaqDNA polymerase (AmpliTaQ, Takara Shuzo Co., Japan, 5 U/ $\mu$ l) 0.2  $\mu$ l were added thereto, and finally deionized water was added to set up total 50  $\mu$ l. PCR was performed by 5 cycles of a cycle consisting of treatment at 95°C for 45 seconds, at 42 °C for 45 seconds and 72°C for 2 minutes, further 35 cycles of a cycle consisting of treatment at 95 °C for 45 seconds, at 50 °C for 45 seconds and 72°C for 2 minutes, and finally allowed to stand at 72°C for 7 minutes. A part of the PCR products was subjected to 2 % agarose gel electrophoresis, stained with ethidium bromide (Nippon Gene Co., Japan), and observed under ultraviolet light to confirm amplification of about 400 bp DNA.

Total amount of PCR product was subjected to electrophoresis with 2 % agarose gel prepared by low melting point agarose (GIBCO BRL Inc., U.S.A.), stained by ethidium bromide, cutting out about 400 bp bands of PCR products by the Delta primer under the UV light, adding distilled water of the same volume of the gel, heating at 65°C for 10 minutes, and completely dissolving the gel. The dissolved gel was centrifuged at 15000 rpm for 5 minutes to separate supernatant solution after adding equal volume of TE saturated phenol (Nippon Gene Co., Japan) and the same separation operation was performed after adding TE saturated phenol : chloroform (1 : 1) solution and chloroform. DNA was recovered from the final solution by ethanol precipitation.

A vector, pCRII vector (Invitrogen Inc., U.S.A., hereinafter designates as pCRII) was used. The vector and the above DNA in molar ratio of 1 : 3 were mixed and DNA was ligated into the vector by using T4 DNA ligase (Invitrogen Inc., U.S.A.). The pCRII, to which DNA was integrated, was subjected to gene transduction into E. coli one shot competent cells (Invitrogen Inc., U.S.A.) and was spread on the semi-solid medium plate of L-Broth (Takara Shuzo Co., Japan) containing ampicillin (Sigma Corp., U.S.A.) 50  $\mu$ g/ml and allowed to stand at 37°C for about 12 hours. The resulting colonies were randomly

selected, inoculated in the L-Broth liquid medium 2 ml containing same concentration of ampicillin and shake cultured at 37°C for about 18 hours. The cultured bacterial cells were recovered and the plasmid was separated by using Wizard Miniprep (Promega Inc., U.S.A.) according to the attached explanation sheet. The plasmid was digested by restriction enzyme EcoRI. Integration of the said PCR product was confirmed by incision of about 400 bp DNA. Base sequence of the incorporated DNA in the confirmed clone was determined by the fluorescent DNA sequencer (Model 373S, Applied System Inc., U.S.A.)

#### Example 2

Cloning of full length novel human Delta-1 and its analysis

A screening of clones having full length cDNA was performed by hybridization from human placenta origin cDNA library (inserted cDNA in  $\lambda$ gt-11, CLONTECH Inc., U.S.A.) in plaques corresponding to  $1 \times 10^6$  plaques. Generated plaques were transferred onto nylon filter (Hybond N+: Amersham Inc., U.S.A.). The transcribed nylon filter was subjected to alkaline treatment [allow to stand for 7 minutes on the filter paper permeated with a mixture of 1.5 M NaCl and 0.5 M NaOH], followed by two neutralizing treatments [allow to stand for 3 minutes on the filter paper permeated with a mixture of 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2) and 1 mM EDTA]. Subsequently, the filter was shaken for 5 minutes in the 2-fold concentrated SSPE solution [0.36 M NaCl, 0.02 M sodium phosphate (pH 7.7) and 2 mM EDTA], washed and air-dried. Then the filter was allowed to stand for 20 minutes on the filter paper, which was permeated with 0.4 M NaOH, shaken for 5 minutes with 5-fold concentrated SSPE solution and washed, then again air-dried. Screening was conducted in the human Delta-1 probe labeled with radioisotope  $^{32}\text{P}$  using these filters.

DNA probe prepared in Example 1 was labeled with  $^{32}\text{P}$  as follows. A DNA fragment was cut out by EcoRI from pCRII, inserted a purified PCR product (about 400 bp) by human Delta-1 primer and determined gene sequence, and was isolated from low melting point agarose gel. The thus obtained DNA fragment was labeled by DNA labeling kit (Megaprime DNA labeling system : Amersham, U.S.A.). The primer solution 5  $\mu$ l and deionized water were added to DNA 25 ng to set up total volume of 33  $\mu$ l, which was treated for 5 minutes in boiling water bath. Reaction buffer solution 10  $\mu$ l containing dNTP,  $\alpha$  -  $^{32}\text{P}$ -dCTP 5  $\mu$ l and T4 DNA polynucleotide kinase solution 2  $\mu$ l were added thereto, treated at 37 °C for 10 minutes in water bath. Subsequently, the mixture was purified by Sephadex column (Quick Spin Column Sephadex G-50 :

Boehringer Mannheim Inc., Germany), then treated for 5 minutes in boiling water bath and ice-cooled for 2 minutes for use.

Hybridization was performed as follows. The prepared filter hereinabove was immersed into the prehybridization solution consisting of SSPE solution, in which final concentration of each component is set at 5-fold concentration, 5-fold concentration of Denhardt's solution (Wako Pure Chemicals, Japan), 0.5 %SDS (sodium dodecyl sulfate, Wako Pure Chemicals, Japan) and salmon sperm DNA (Sigma, U.S.A.) 10  $\mu$ g/ml denatured by boiling water, and shaken at 65°C for 2 hours, then the filter was immersed into the hybridization solution of the same composition with the above prehybridization solution with the <sup>32</sup>P-labeled probe above mentioned and shaken at 65°C for 2 hours for 16 hours to perform hybridization.

The filter was immersed into SSPE solution containing 0.1 % SDS, shaken at 55°C and washed twice, further immersed into 10-fold dilution of SSPE solution containing 0.1% SDS and washed four times at 55°C. An autoradiography of the washed filter was performed using intensified screen. Clones of strongly exposed part were collected and the plaques obtained were again spread and screened by the same method hereinbefore to separate complete single clones.

The thus isolated phage clones were seven clones. Phage of all of these clones was prepared to about  $1 \times 10^9$  pfu, purified the phage DNA, digested by restriction enzyme EcoRI and inserted into pBluescript (Stratagene Inc., U.S.A.) which was digested EcoRI in the same way. DNA sequences of the both ends of these clones were analyzed by DNA sequencer. Three clones of D5, D6 and D7 were the clone containing DNA sequence from No. 1 to 2244 in the sequence listing, SEQ ID NO: 8. A clone D4 was a clone containing DNA sequence from No. 999 to 2663 in the sequence listing, SEQ ID NO: 8. The clones D5 and D4 prepared the deletion mutant by using kilosequence deletion kit (Takara Shuzo Co., Japan) according to a description of the attached paper. Full-length cDNA base sequence of the present invention was determined using the DNA sequencer from both direction of 5'-direction and 3'-direction.

By applying with XhoI site at No. 1214 in DNA sequence in the sequence listing, SEQ ID NO: 8, D4 and D5 were digested by restriction enzyme XhoI to prepare plasmid pBSDel-1 containing full length of DNA sequence in the sequence listing, SEQ ID NO: 8.

### Example 3

Cloning of human Serrate-1 specific PCR product and determination of



base sequence

A mixed primer, which corresponded to amino acid sequence conserved in *Drosophila* Serrate and rat Jagged, i.e. sense primer SRTS 1 (the sequence listing, SEQ ID NO:16) and antisense primer SRTA2 (the sequence listing, SEQ ID NO:17), was used. Preparation was conducted by the same way as described in Example 1.

Amplification by PCR using these primers was performed as follows. To the human fetal brain originated cDNA mixed solution hereinbefore 1  $\mu$ l was added 10 $\times$  buffer solution (described in Example 1) 5  $\mu$ l, said dNTP mixture 4  $\mu$ l, sense primer SRTS1 (100 pmol/ $\mu$ l) 5  $\mu$ l and antisense primer SRTA2 (100 pmol/ $\mu$ l) 5  $\mu$ l specific to Serrate-1 homologue hereinbefore, and said TaqDNA polymerase 0.2  $\mu$ l, and finally added deionized water to set up total volume 50  $\mu$ l. The mixture was treated for 5 cycles of a cycle consisting of at 95°C for 45 seconds, at 42°C for 45 seconds and 72°C for 2 minutes, and 35 cycles of a cycle consisting of at 95°C for 45 seconds, at 50°C for 45 seconds and 72°C for 2 minutes, and finally allowed to stand at 72°C for 7 minutes to perform PCR. A part of the PCR product was subjected to 2 % agarose gel electrophoresis, stained by ethidium bromide, and observed under ultraviolet light to confirm amplification of about 500 bp cDNA.

Total amount of PCR product was subjected to electrophoresis with 2 % agarose gel prepared by low melting point agarose, stained by ethidium bromide, cutting out about 500 bp bands under the UV light, adding distilled water of the equal volume of the gel, heating at 65°C for 10 minutes, and completely dissolving the gel. The dissolved gel was centrifuged at 15000 rpm for 5 minutes to separate supernatant solution after adding equal volume of TE saturated phenol and the same separation operation was performed after adding TE saturated phenol : chloroform (1 : 1) solution and chloroform. DNA was recovered from the final solution by ethanol precipitation.

A vector, pCRII vector was used. The vector and the above DNA were mixed in molar ratio of 1 : 3 and DNA fragment was ligated into the vector pCRII by the same method in Example 1. The pCRII, to which DNA was integrated, was subjected to gene transduction into *E. coli*. The resulting colonies were randomly selected and were inoculated in liquid medium L-Broth 2 ml containing the same concentration of ampicillin and shake cultured at 37°C for about 18 hours. The cultured bacterial cells were recovered and the plasmid was separated by using the Wizard Miniprep according to the attached explanatory sheet. The plasmid was digested by restriction enzyme EcoRI. Integration of the said PCR product was confirmed by incision of about 500 bp DNA. Base

sequence of the incorporated DNA in the confirmed clone was determined by the fluorescent DNA sequencer.

#### Example 4

Cloning of full length novel human Serrate-1 and its analysis

A screening of clones having full length cDNA was performed by hybridization from the human placenta origin cDNA library hereinbefore in plaques corresponding to  $1 \times 10^6$  plaques. Preparation of the filter was performed by the same method as described in Example 2. Screening was conducted in the human Serrate-1 probe labeled with radioisotope  $^{32}\text{P}$  using the filter.

The above DNA probe labeled with  $^{32}\text{P}$  was prepared by a method described in Example 2, and hybridization, washing of the filter and isolation of the clone were performed by the description in Example 2.

The thus isolated phage clones were 22 clones. Phage of all of these clones was prepared to about  $1 \times 10^9$  pfu, purified the phage DNA, digested by restriction enzyme EcoRI and inserted into pBluescript which was digested EcoRI in the same way. DNA sequences of the both ends of these clones were analyzed by DNA sequencer. Two clones of S16 and S20 were the clone containing DNA sequence from No. 1 to 1873 in the sequence listing, SEQ ID NO: 10. Two clones S5 and S14 were the clones containing DNA sequence from No. 990 to 4005 in the sequence listing, SEQ ID NO: 10. These clones prepared the deletion mutants by using the kilosequence deletion kit according to a description of the attached leaflet. The cDNA base sequence coding the polypeptide of the present invention was determined using the DNA sequencer from both direction of 5'-direction and 3'-direction.

By applying with BglII site at No. 1293 in DNA sequence in the sequence listing, SEQ ID NO: 10, S20 and S5 were digested by restriction enzyme BglII, and DNA of gene sequence from No. 1 to 4005 in the sequence listing SEQ ID NO: 10 was subcloned in E. coli vector pBluescript. This plasmid is named as pBSSRT.

Since the termination codon was not found in the C-terminal and the intracellular region coding C-terminal amino acids was not cloned, cloning of the full length gene was performed using the 3' RACE system kit, GIBCO-BRL, U.S.A., according to the description of the attached leaflet. The cloning of cDNA gene for 3'-direction was performed in polyA<sup>+</sup> RNA (CLONTECH Inc., U.S.A.) originated from human placenta to determine the gene sequence.

The thus cloned three gene fragments by applying with BglII site in DNA sequence No. 1293 and AccI site in DNA sequence No. 3943 and a plasmid

containing full length of DNA sequence in the sequence listing, SEQ ID NO: 5 were inserted between EcoRI and XbaI in the multi-cloning site of pUC18 to prepare pUCSR-1 containing full length gene of human Serrate-1. This gene sequence as well as its amino acid sequence is shown in the sequence listing, SEQ ID NO: 10.

#### Example 5

##### Preparation of expression vectors of human Delta-1

Using the gene consisting of DNA sequence described in the sequence listing, SEQ ID NO: 7, expression vectors of human Delta-1 protein mentioned in the following 1) - 5) were prepared. Addition of restriction enzyme sites and insertion of short gene sequence were performed using ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene Inc., U.S.A.) according to the operating manual.

##### 1) Expression vector of soluble human Delta-1 protein (HDEX)

The cDNA coding polypeptide of amino acid sequence from No.1 to 520 in the sequence listing, SEQ ID NO: 3 was ligated with expression vector pMKITNeo containing SR $\alpha$  promoter and neomycin resistance gene to prepare expression vector.

For preparation of expression vector of human Delta-1, in order to stable expression from gene product, EcoRI site was added in the 20 bp upper stream for 5'-direction of the initiation codon (gene sequence No. 179 in the sequence listing, SEQ ID NO: 8). Using the above Mutagenesis Kit, a plasmid pBSDel-1, which contained DNA sequence in sequence listing, SEQ ID NO: 8 and full length cDNA of human Delta-1 were set as the template, and oligonucleotides having gene sequence in sequence listing, SEQ ID NO:18 and SEQ ID NO:19 was set as the primers. Then DNA adding EcoRI site in the 20 bp upper stream for 5'-direction was prepared. Hereinafter this plasmid is designated as pBS/Eco-Delta.

The pBS/Eco-Delta was used as a template. In order to add the termination codon and restriction enzyme MluI site after a C-terminal position, using the Mutagenesis Kit, and setting oligonucleotides having gene sequences in the sequence listing, SEQ ID NO:20 and SEQ ID NO: 21 as primers, addition of the termination codon and MluI site were performed. The resulted vector was digested by EcoRI and MluI, and about 1600 bp splitted gene fragment was ligated in pMKITNeo, which was treated by the same restriction enzyme, to construct the expression vector. This vector was designated as pHDEX.

2) Expression vector of FLAG chimera protein of soluble human  
Delta-1 (HDEXFLAG)

The cDNA coding chimera protein, to which cDNA coding FLAG sequence was added to the C-terminal of polypeptide from No. 1 to 520 of amino acid sequence in the sequence listing, SEQ ID NO: 3, was ligated to the expression vector pMKITNeo containing SR $\alpha$  promoter and neomycin resistance gene to prepare the expression vector.

Using pBS/Eco-Delta as template, FLAG sequence was added in the extracellular C-terminal, i.e. after Gly at No. 520 in the sequence listing, SEQ ID NO: 3. In order to add the termination codon and restriction enzyme MluI site, using the Mutagenesis Kit, and setting oligonucleotides having gene sequence in the sequence listing, SEQ ID NO:22 and SEQ ID NO:21 as primers, a gene coding FLAG sequence and termination codon and MluI site were added in the C-terminal. This vector was digested by EcoRI and MluI, and about 1700 bp splitted gene fragment was ligated to the similarly restriction enzyme treated pMKITNeo to construct the expression vector. This vector was designated as pHDEXFLAG.

3) Expression vector of IgG1Fc chimera protein of soluble human  
Delta-1 (HDEXIg)

A gene sequence coding polypeptide, to which amino acid sequence of Fc region below the hinge part of human IgG1 was added to the C-terminal of polypeptide having amino acid sequence in the sequence listing, SEQ ID NO: 3.

Preparation of fused protein with immunoglobulin Fc protein was performed according to the method of Zettlmeissl et al. (Zettlmeissl et al., DNA cell Biol., 9, 347-354, 1990). A gene using genome DNA with intron was applied and the said gene was prepared by using PCR. Human genome was used as a template. An oligonucleotide of the sequence in the sequence listing, SEQ ID NO: 23 with restriction enzyme BamHI site and an oligonucleotide of the sequence in the sequence listing, SEQ ID NO: 24 with restriction enzyme XbaI site were used as primers. PCR was performed using the primers and human genomic DNA as template. About 1.4 kbp band was purified, treated by restriction enzyme BamHI and XbaI (Takara Shuzo Co., Japan), and genes were ligated to pBluescript, which was similarly treated by restriction enzyme, by

using T4 DNA ligase to prepare subcloning. Later, the plasmid DNA was purified and sequenced to confirm gene sequence, then the said gene sequence was confirmed as genomic DNA in the hinge region of heavy chain of the human IgG1. (The sequence is referred to Kabat et al., Sequence of Immunological Interest, NIH Publication No. 91 - 3242, 1991). Hereinafter this plasmid is designated as pBSIgFc.

Using the said pBS/Eco-Delta as template, and using the Mutagenesis Kit, restriction enzyme BamHI site was added in the extracellular C-terminal, i.e. after Gly at No. 520 in the sequence listing, SEQ ID NO: 3. Furthermore, in order to add restriction enzyme XbaI and MluI sites to the downstream, and setting the oligonucleotides having gene sequence in the sequence listing, SEQ ID NO: 25 and SEQ ID NO: 26, using the Mutagenesis Kit, BamHI, XbaI and MluI sites were added. This vector digested by XbaI and BamHI and about 1200 bp of gene fragment digested from the above pBSIgFc by XbaI and BamHI were ligated to prepare vector containing gene fragments coding the final objective soluble human Delta-1 IgG1Fc chimera protein. Finally, this vector was digested by EcoRI and MluI and about 3000 bp splitted gene fragments were ligated with the similarly restriction enzyme treated pMKITNeo to construct the expression vector. This vector was designated as pHDEXIg.

#### 4) Expression vector of full length human Delta-1 protein (HDF)

The cDNA coding polypeptide from No. 1 to 702 of amino acid sequence in the sequence listing, SEQ ID NO: 4, was ligated to the expression vector pMKITNeo containing SR $\alpha$  promoter and neomycin resistance gene to prepare the expression vector.

In order to add the termination codon in C-terminal of the full length sequence, i.e. after Val at No. 702 in the sequence listing, SEQ ID NO: 4 and restriction enzyme MluI site, using the Mutagenesis Kit and pBS/Eco-Delta as template and setting oligonucleotides having gene sequence in the sequence listing, SEQ ID NO: 27 and SEQ ID NO: 28 as primers, the termination codon and MluI site were added in the C-terminal. This vector was digested by EcoRI and MluI, and about 2200 bp splitted gene fragment was ligated to the similar

restriction enzyme-treated pMKITNeo to construct the expression vector. This vector was designated as pHDF.

5) Expression vector of FLAG chimera protein (HDFLAG) of full length human Delta-1

The cDNA coding chimera protein, to which cDNA coding FLAG sequence was added to the C-terminal of polypeptide from No.1 to 702 of amino acid sequence in the sequence listing, SEQ ID NO: 4, was ligated to the expression vector pMKITNeo containing SR $\alpha$  promoter and neomycin resistance gene to prepare the expression vector.

In order to add FLAG sequence in the C-terminal, the termination codon and restriction enzyme MluI site, setting oligonucleotides having gene sequence in the sequence listing, SEQ ID NO: 29 and SEQ ID NO: 28 as primers and using pBS/Eco-Delta as template, a gene coding FLAG sequence and termination codon and MluI site were added in the C-terminal. From this vector, DNA coding full length of human Delta-1 was cloned in E. coli vector pUC19 to prepare vector pUCDL-1F coding full length of human Delta-1. This vector was digested by EcoRI and MluI, and about 2200 bp splitted gene fragments were ligated to the similar restriction enzyme treated pMKITNeo to construct the expression vector. This vector was designated as pHDFLAG.

Example 6

Preparation of expression vectors of human Serrate-1

Using the gene consisting of DNA sequence described in the sequence listing, SEQ ID NO: 10 expression vectors of human Serrate-1 protein mentioned in the following 6) - 10) were prepared. Addition of restriction enzyme sites and insertion of short gene sequence were performed by using the ExSite PCR-Based Site-Directed Mutagenesis Kit as well as according to the operating manual.

6) Expression vector of soluble human Serrate-1 protein (HSEX)

The cDNA coding polypeptide of amino acid sequence from No. 1 to 1036 in the sequence listing, SEQ ID NO: 6 was ligated with expression vector pMKITNeo to prepare expression vector.

For preparation of expression vector of polypeptide expression cells having amino acid sequence from No. 1 to 1036 in the sequence listing, SEQ ID NO: 6, in order to express gene product more stably EcoRI site was added in the 10 bp upper stream region for 5' - direction of the initiation codon (gene sequence No. 409 in the sequence listing, SEQ ID NO: 10). Using the above Mutagenesis Kit, a plasmid pBSSRT, which contained cDNA of human Serrate-1

from No.1 to 4005 of DNA sequence in the sequence listing, SEQ ID NO:10, was set as the template, and oligonucleotide having gene sequence in sequence listing, SEQ ID NO: 30 and oligonucleotide having gene sequence in sequence listing, SEQ ID NO: 31 were set as the primers. Then DNA adding EcoRI site in the 10 bp upper stream for 5' - direction was prepared.

The thus prepared vector (hereinafter designates as pBS/Eco-Serrate-1) was used as a template. In order to add the termination codon and further restriction enzyme MluI site in the extracellular C-terminal region, i.e. C-terminal of polypeptide in the sequence listing, SEQ ID NO: 6, using the Mutagenesis Kit, and setting oligonucleotide having gene sequence in the sequence listing, SEQ ID NO: 32 and oligonucleotide having gene sequence in the sequence listing, SEQ ID NO: 33 as primers, the termination codon and MluI site were added. The resulting vector was digested by EcoRI and MluI, and about 3200 bp splitted gene fragment was ligated in pMKITNeo, which was treated by the same restriction enzyme, to construct the expression vector. This vector was designated as pHSEX.

7) Expression vector of FLAG chimera protein of soluble human Serrate-1 (HSEXFLAG)

The cDNA coding FLAG chimera protein, which had FLAG sequence in the C-terminal of polypeptide from No. 1 to 1036 of amino acid sequence in the sequence listing, SEQ ID NO: 6, was ligated to the expression vector pMKITNeo containing SR $\alpha$  promoter and neomycin resistance gene to prepare the expression vector.

Using pBS/Eco-Serrate-1 as a template, FLAG sequence was added in the extracellular C-terminal, i.e. the C-terminal of polypeptide in the sequence listing, SEQ ID NO: 6. In order to add the termination codon and further restriction enzyme MluI site, using the Mutagenesis Kit, and setting oligonucleotide having gene sequence in the sequence listing, SEQ ID NO: 34 and oligonucleotide having gene sequence in the sequence listing, SEQ ID NO: 33 as primers, a gene coding FLAG sequence and termination codon and MluI site were added in the C-terminal. This vector was digested by EcoRI and MluI, and about 3200 bp splitted gene fragment was ligated to the similarly restriction enzyme treated pMKITNeo to construct the expression vector. This vector was designated as pHSEXFLAG.

8) Expression vector of IgG1Fc chimera protein of soluble human Serrate-1 (HSEXIg)

A gene sequence coding polypeptide, to which amino acid sequence of Fc region below the hinge part of human IgG1 was added to the C-terminal of

polypeptide having amino acid sequence in the sequence listing, SEQ ID NO: 6.

In order to add restriction enzyme BamHI site in the extracellular C-terminal, i.e. after the polypeptide having the sequence in the sequence listing, SEQ ID NO: 6 and further restriction enzyme XbaI and MluI sites to its downstream, BamHI, XbaI and MluI sites were added Using pBS/Eco-Serrate-1 as a template by the Mutagenesis Kit, using oligonucleotide having gene sequence in the sequence listing, SEQ ID NO: 35 and oligonucleotide having gene sequence in the sequence listing, SEQ ID NO: 36 as primers. This vector digested by XbaI and BamHI and about 1200 bp of gene fragment digested from the above pBSIgFc by XbaI and BamHI were ligated to finally prepare a vector, which contained gene fragments coding IgG1Fc chimera protein of the soluble human Serrate-1. Finally, this vector was digested by EcoRI and MluI, and splitted about 4400 bp gene fragment was ligated to pMKITNeo to construct the expression vector. This vector was designated as pHSEXIg.

9) Expression vector of full length human Serrate-1 protein (HSF)

The cDNA coding polypeptide from No. 1 to 1187 of amino acid sequence in the sequence listing, SEQ ID NO: 7 was ligated with expression vector pMKITNeo containing SR $\alpha$  promoter and neomycin resistance gene to prepare expression vector.

For preparation of the full length expression vector about 900 bp splitted gene fragment from pBS/Eco-Serrate-1 digested by restriction enzyme EcoRI and BglII, and pUCSR-1 digested by the same restriction enzyme were ligated, and a vector pUC/Eco-Serrate-1 coding full length gene of human Serrate-1 was prepared.

In order to add the termination codon to the site after Val at No.1187 in the sequence listing, SEQ ID NO: 7, and further add the restriction enzyme MluI site, using the Mutagenesis Kit, the termination codon and MluI site were added to the C-terminal using oligonucleotides having gene sequence in the sequence listing, SEQ ID NO:37 and SEQ ID NO:38 as primers and the pBS/Eco-Serrate-1 as a template. The resulting vector was digested by EcoRI and MluI, and about 3700 bp splitted gene fragments were ligated in pMKITNeo, which was treated by the same restriction enzyme, to construct the expression vector. This vector was designated as pHSF.

10) Expression vector of FLAG chimera protein of full length human Serrate-1 (HSFLAG)

The cDNA coding chimera protein, to which cDNA coding FLAG sequence was added in the C-terminal of polypeptide from No. 1 to 1187 of amino acid sequence in the sequence listing, SEQ ID NO: 7, was ligated to the expression



vector pMKITNeo containing SR $\alpha$  promoter and neomycin resistance gene to prepare the expression vector.

In order to add FLAG sequence in the C-terminal the termination codon and further restriction enzyme MluI site, setting oligonucleotides having gene sequence in the sequence listing, SEQ ID NO: 39 and SEQ ID NO: 38 as primers, using pBS/Eco-Serrate-1 as a template a gene coding FLAG sequence, the termination codon and the MluI site were added in the C-terminal as same as similar manner. This vector was digested by EcoRI and MluI, and about 3700 bp splitted gene fragments were ligated to the similarly restriction enzyme treated pMKITNeo to construct the expression vector. This vector was designated as pHSFLAG.

#### Example 7

Expression and Gene transfer of the expression vectors into cells

The expression vectors prepared in Examples 5 and 6 were transduced into COS-7 cell (obtained from RIKEN Cell Bank, Physical and Chemical Research Institute, Japan, RCB0539).

Cell culture before gene transduction was performed by culturing in D-MEM (Dulbecco modified Eagle's medium, GIBCO-BRL Inc., U.S.A.) 10 % FCS. On a day before gene transduction, medium of cells was changed to set cell counts  $5 \times 10^5$  cells/ml and cultured for overnight. On the day of gene transduction, cells were sedimented by centrifugation, centrifugally washed twice with PBS(-) and prepared the cells to  $1 \times 10^7$  cells/ml in 1 mM MgCl<sub>2</sub> and PBS(-). Gene transfer was performed by electroporation using gene transduction device Gene-pulsar (Bio-Rad Inc., U.S.A.). The above cell suspension 500  $\mu$ l was collected in the cell for electroporation (0.4 cm), added expression vector 20  $\mu$ g, and allowed to stand in ice for 5 minutes. Electroporation was performed under the condition 3  $\mu$ F, 450 V twice, during the twice electroporation cell mixture was allowed to stand at room temperature. After 5 minutes stayed in ice, cells were spread in the culture medium, diameter 10 cm previously added 10 ml of medium, and cultured at 37°C in 5 % carbon dioxide incubator.

The next day, the culture supernatant solution was removed, washed the cells adhered to the dish twice with PBS(-) 10 ml. In case of expression vector pHDEX, pHDEXFLAG, pHDEXIg, pHSEX, pHSEXFLAG, and pHSEXIg, serum-free D-MEM 10 ml was added and cultured for 7 days. Culture supernatant solution was recovered and was replaced the buffer to PBS(-) by Centricon 30 (Amicon Inc., U.S.A.) and simultaneously the solution was concentrated to 10-fold to

obtain cell culture supernatant solution.

In case of pHDF, pHDFLAG, pHSF, and pHSFLAG, medium was changed by D-MEM containing 10 % FCS, and cultured further 3 days to prepare cell lysate. Thus,  $2 \times 10^6$  cells were suspended in the cell lysis buffer [50 mM Hepes (pH 7.5), 1 % Triton X100, 10 % glycerol, 4 mM EDTA, 50  $\mu$ g/ml Aprotinin, 100  $\mu$ M Leupeptin, 25  $\mu$ M Pepstatin A and 1 mM PMSF] 200  $\mu$ l, allowed to stand in ice for 20 minutes and centrifuged at 14000 rpm for 20 minutes to remove supernatant solution to obtain cell lysate.

Expression of proteins were detected by Western blotting.

Concentrated cultured supernatants or cell lysates were subjected to SDS-PAGE using an electrophoresis tank and polyacrylamide gel for SDS-PAGE (gradient gel 5 - 15 %) (ACI Japan Inc., Japan) according to the manufacturer's construction. Samples were prepared by treatment in boiling water for 5 min. with 2-mercaptoethanol (2-ME) for reduction, and non-reduced condition without taking the above treatment. As a marker, Rainbow Marker (high molecular weight, Amersham Inc.) was used. Sample buffer solution and electrophoresis buffer were prepared with reference to the attached leaflet. When the SDS-PAGE was finished, acrylamide gel was transcribed to PVDF membrane filter (BioRad Inc., U.S.A.) using the Mini Trans Blot Cell (BioRad Inc.).

The thus prepared filter was shaken overnight at 4°C in the Blockace (Dainippon Pharm. Co., Japan), TBS-T [20 mM Tris, 137 mM NaCl (pH 7.6) and 0.1 % Tween 20] to blocking. According to the explanation of the attached leaflet of ECL Western blotting detection system (Amersham Inc., U.S.A.); in case that the objective protein was human Delta-1 origin, anti-human Delta-1 mouse monoclonal antibody described in Example 9 was used as primary antibody; in case that protein was human Serrate-1 origin, anti-human Serrate-1 mouse monoclonal antibody described in Example 9 was used as primary antibody; and in case that protein was FLAG chimera, anti-FLAG M2 mouse monoclonal antibody (Eastman Kodak, U.S.A.) was used as primary antibody, and peroxidase labeled anti-mouse Ig sheep antibodies (Amersham Inc., U.S.A.) was reacted. In case of IgG chimera, peroxidase labeled anti-human Ig sheep antibodies (Amersham

Inc., U.S.A.) was reacted.

Reaction time for antibodies was 1 hour at room temperature, and at an interval of each reaction, washing was performed by shaking in TBS-T at room temperature for 10 minutes for three times. After the final washing, the filter was immersed in the reaction solution of ECL-Western blotting detection system (Amersham Inc., U.S.A.) for 5 minutes, and wrapped in polyvinylidene chloride wrap to expose X-ray film.

As the result, in the sample with treatment of reduction, the bands showing protein obtained by transduction of pHDEX and pHDEXFLAG was detected about 65 kD ; protein obtained by transduction of pHDEXIg was detected about 95 kD, and protein obtained by transduction of pHDF and pHDFFLAG was detected about 85 kD. In the non-reduced sample, the bands showing protein obtained by transduction of pHDEXIg was detected slightly smeared bands at 120 kD to 200 kD, mainly about 180 kD, which showed about 2-fold of the reduction stage, consequently, dimer was formed.

And also, in the sample with treatment of reduction, the bands showing protein obtained by transduction of pHSEX and pHSEXFLAG was detected about 140 kD ; protein obtained by transduction of pHSEXIg was detected about 170 kD, and protein obtained by transduction of pHSEF and pHSEFLAG was detected about 150 kD. In the non-reduced sample, the bands showing protein obtained by transduction of pHSEXIg was detected slightly smeared bands at 250 kD to 400 kD, mainly about 300 kD, which showed about 2-fold of the reduction stage, consequently, dimer was formed.

In these experiments, however cell lysate and cultured supernatant of COS-7 cells, to which pMKITNeo vector was transduced as a control was tested., no bands reacted against anti-human Delta-1 mouse monoclonal antibody, anti-human Serrate-1 mouse monoclonal antibody, anti-FLAG antibody, and anti-human Ig antibody were detected.

Therefore, this ten-expression vector can produce the objective polypeptides.

### Example 8

Purification of soluble human Delta-1 and human Serrate-1 proteins of gene transduction cells

Cultured supernatant of COS-7 cells consisting of HDBXFLAG, HDBXlg, HSEXFLAG and HSEXlg, all of which expression was detected by a method in Example 7, were prepared on large scale, and each chimera protein was purified by affinity column chromatography.

In case of HDEXFLAG and HSEXFLAG, 2 liter of the cultured supernatant obtained by the method in Example 7 was passed through a column packed with Anti-FLAG M2 Affinity Gel (Eastman Kodak, U.S.A.). The chimera protein was adsorbed in a column by a reaction of affinity of anti-FLAG antibody of the gel and FLAG sequence of the chimera protein. Column, inner diameter 10 mm, disposable column (BioRad Inc., U.S.A.) was used with packing the above gel 5 ml. A circulation system consisting of medium bottle → column → peristaltic pump → medium bottle was set up. The circulation was run by a flow 1 ml/min. for 72 hours. Thereafter the column was washed with PBS (-) 35 ml and eluted by 0.5 M Tris-glycine (pH 3.0) 50 ml. The eluate of 25 fractions, each 2 ml, was collected into the tube, and each fraction was neutralized by 200  $\mu$ l of 0.5 M Tris-HCl (pH 9.5) previously added in each tube.

The eluate fraction, each 10  $\mu$ l of the secretor FLAG chimera protein which was purified by the above method was subjected to reduction treatment described in Example 7. SDS-PAGE electrophoresis by 5-10 % gradient polyacrylamide gel was performed. After finishing the electrophoresis, silver staining was conducted by using Wako silver stain kit II (Wako Pure Chemicals, Japan) according to the explanation of the attached leaflet. Fractions from No. 4 to 8 showed detectable bands in HSFLAG. The size is identical with the result of Western blotting of anti-FLAG antibody obtained in Example 6 in both of HDEXFLAG and HSEXFLAG. Therefore, purified HDEXFLAG and HSEFLAG were obtained.

In the IgG1Fc chimera protein, i. e. HDEXlg and HSEXlg, the cultured supernatant solution 2 liter was adsorbed in Protein A Sepharose

colulnn (Pharmacia Inc., Sweden) according to the same method as of FLAG chimera protein to collect the eluate fractions. Using a part of eluate as same as in FLAG chimera protein, a determination of the eluate fraction, identification of the size and detection of the purity were performed by SDS-PAGE electrophoresis and silver staining in the reduced condition. Therefore, the eluate fraction from No. 4 to 15 were the detected bands. The size thereof is identical with the result of Western blotting using anti-human Ig antibody in both of HDEXIg and HSEXIg. Therefore, purified HDEXIg and HSEXIg were obtained.

#### Example 9

Preparation of antibodies recognizing human Delta-1 and human Serrate-1

HDEXFLAG and HSEXFLAG, purified by the method in Example 8, were used as immunogen, and rabbits were immunized. After assaying antibody titer, whole blood was collected and serum was obtained. Anti-human Delta-1 rabbit polyclonal antibody and anti-human Serrate-1 rabbit polyclonal antibody were purified by using the econopack serum IgG purification kit (BioRad Inc., U.S.A. ) with reference to the attached explanation leaflet.

HDEXFLAG and HSEXFLAG purified by a method described in Example 8 were used as immunogens, and mouse monoclonal antibodies were prepared according to the explanation of the textbook. The purified HDEXFLAG or HSEXFLAG was administered in Balb/c mice (Nippon SLC CO., Japan) separately, 10  $\mu$ g/mouse, immunized intracutaneously and subcutaneously. After second immunization increased serum titer was confirmed by collecting blood ophthalmologically, the third immunization was performed. Subsequently, the spleen of mice was collected and fused with mouse myeloma cells P3  $\times$  63Ag8 (ATCC TIB9) using polyethylene glycol. Hybridoma was selected by HAT medium (Immunological and Biological Research Institute, Japan), and the hybridoma strains which produced antibody specifically recognizing extracellular region of human

Delta-1 or human Serrate-1 in the medium, were isolated by enzyme immunoassay. The hybridoma strains producing mouse monoclonal antibody, which specifically recognized human Delta-1 or human Serrate-1, were established.

Anti-human Delta-1 monoclonal antibody and anti-human Serrate-1 monoclonal antibody were purified and prepared by using Mab Trap GII (Pharmacia Inc., Sweden) and according to the explanation of the leaflet, from the supernatant of the thus established hybridoma.

Affinity column was prepared by using these monoclonal antibodies. Preparation of the affinity column was performed according to the explanation attached to the CNBr activated Sephadex 4B (Pharmacia Inc., Sweden). A column,  $2\text{ cm}^2 \times 1\text{ cm}$ , containing gel 2 ml, was prepared.

A concentrated solution of the supernatant of the cultured COS-7 cells, to which pHDEX was gene transduced, was passed through the column for which anti-human Delta-1 monoclonal antibody was bound. A concentrated solution of the supernatant of the cultured COS-7 cells, to which pHSEX was gene transduced, was passed through the column, for which anti-human Serrate-1 monoclonal antibody was bound. Each supernatant solution was passed at 20 ml/hr, subsequently PBS (-) 15 ml was passed at the same flow rate and washed the column. Finally, the products were eluted by a mixture of 0.1 M sodium acetate and 0.5 M NaCl (pH 4.0). The eluate, each 1 ml fraction, was collected, and was neutralized by adding 1M Tris-HCl (pH 9.1) 200  $\mu$ l for each fraction.

SDS-PAGE of each purified protein was conducted under reduced condition according to the method described in Example 8, followed by silver staining and Western blotting to estimate molecular weight. HDEX, about 65 kD, was purified from concentrated supernatant of the cultured COS-7 cells, to which pHDEX was gene transduced, and HDSEX, about 140 kD, was purified from concentrated supernatant of the cultured COS-7 cells, to which pHSEX was gene transduced. Consequently, human Delta-1 and human Serrate-1 can be purified by these affinity columns.

Example 10

Effects of HDEXIg and HSEXIg to colony formation of blood undifferentiated cells

In order to observe physiological action of HDEXIg and HSEXIg on blood undifferentiated cells, CD34 positive cells were cultured in the serum-free semi solid medium in the presence of HDEXIg and HSEXIg and known cytokines, and number of colony forming cells were observed.

Human umbilical cord blood or adult human normal bone marrow blood was treated by the silica solution (Immunological and Biological Research Institute, Japan) according to the attached explanation leaflet. Thereafter the low density cellular fraction ( $< 1.077$  g/ml) was fractionated by densitometric centrifugation of Ficoll pack (Pharmacia Inc., Sweden) to prepare mononuclear cells. CD34 positive cells of human umbilical cord blood or human normal bone marrow blood was isolated from the mononuclear cells.

Separation of CD34 positive cells was performed by using Micro-Selector System (AIS Inc., U.S.A.) or Dynabeads M-450 CD34 and DETACHA-BEADS CD34 (DynaI Inc., Norway) according to attached explanation leaflets. After separation, the purity was measured as follows. Cells were stained by FITC labeled CD34 antibody HPCA2 (Beckton-Deckinson Inc., U.S.A.) and examined by a flow-cytometer (FACSCalibur, Beckton-Deckinson, U.S.A.). Purity above 85 % was confirmed for use.

The thus isolated CD34 positive cells were suspended homogeneously to form 400 cells/ml of the medium hereinbelow, and spread in the 35 mm dish (Falcon Inc., U.S.A.), then cultured for 2 weeks in carbon dioxide incubator at 37°C under 5 % carbon dioxide, 5 % oxygen, 90 % nitrogen and 100 % humidity. The formed blood colonies were counted under the invert microscope.

A medium used is  $\alpha$ -medium (GIBCO-BRL, U.S.A.), containing 2 % deionized bovine serum albumin (BSA, Sigma, U.S.A.), 10  $\mu$ g/ml human insulin (Sigma, U.S.A.) 200  $\mu$ g/ml transferrin (Sigma, U.S.A.),  $10^{-5}$ M 2-mercaptoethanol (Nakarai Tesk Co., Japan), 160  $\mu$ g/ml soybean lectin

(Sigma, U.S.A.), 96  $\mu$ g/ml cholesterol (Sigma, U.S.A. ) and 0.9 % methylcellulose (Wako Pure Chemicals, Japan).

To the above medium under the following three conditions of cytokines, human Delta-1 extracellular Ig chimera protein (HDEXIg) or human Serrate-1 extracellular Ig chimera protein (HSEXIg) were added to the final concentration of 1  $\mu$ g/ml. For control, human IgG1 (Ahens Research and Technology Inc., U.S.A.) was added with the same concentration in order to observe effect of IgGFc region.

Conditions of cytokines are as follows.

1 : 100  $\mu$ g/ml, human SCF (Intergen Inc., U.S.A.), 10 ng/ml human IL-3 (Intergen Inc., U.S.A.), 100 ng/ml human IL-6 (Intergen Inc., U.S.A. )

2 : 100 ng/ml human SCF, 10 ng/ml human IL-3, 4 ng/ml human thrombopoietin (Pepro Tech Inc., U.S.A.)

3 : 100 ng/ml human SCF, 10 ng/ml human IL-3, 100 ng/ml human IL-6 , 2U/ml Epo (Chugai Seiyaku Co., Japan) 10 ng/ml human G-CSF (Chugai Seiyaku Co., Japan)

Results are shown in Fig. 2. In Fig.2, A is a case of human Delta-1 extracellular Ig chimera protein (HDEXIg), and B is a case of human Serrate-1 extracellular Ig chimera protein (HSEXIg). For A and B, each different origin human umbilical cord blood CD34 positive cell was used. The vertical axis: number of colonies. White: control, black: HDEXIg or HSEXIg. Both HDEXIg and HSEXIg have suppressive action of colony formation. No differences of the activities on the types of colonies were noted. Therefore, the molecular of the present invention has suppressive action for colony formation against colony forming cells of blood undifferentiated cells, i.e. differentiation-suppressive action. Comparison with or without SCF on the activity indicated that the suppressive action tended to occur only in the presence of SCF.

Dose-dependent manner of the activity was studied. Comparison with dimer HSEXIg and monomer HSEXFLAG was performed. Result is shown in Fig.3. Concentration in this case is indicated as molar concentration. For comparison with dimer and monomer, dimer HSEXIg was indicated by



exact two molar concentrations, and was plotted equivalent molar concentration of the human Serrate-1. Vertical axis indicates colony forming counts and horizontal axis indicates molar concentration. Colony forming counts without Notch ligand were plotted on the vertical axis in the zero concentration. For comparison, colony forming counts of human IgG1 1  $\mu$ g/ml, was about 100 colonies.

This result indicated that HSEXIg and HSEXFLAG suppressed colony formation in dose-dependent manner. Activity of dimer HSEXIg was stronger than the monomer. A monomer HSEXFLAG showed stimulative action for colony formation in the low concentration area.

#### Example 11

Actions of HDEXIg and HSEXIg on long term liquid culture of colony forming blood undifferentiated cells

For observing physiological action of HDEXIg and HSEXIg on the blood undifferentiated cells, umbilical cord blood CD34 positive cells were culture for long term in the serum-free liquid medium in the presence of HDEXIg or HSEXIg and known cytokines, and numbers of colony forming cells were observed.

The umbilical cord blood mononuclear CD34 positive cells separated by a method described in Example 10 were liquid cultured at 1000 cells/well in the 24 well cell culture plate (Falcon Inc., U.S.A.). Culture was performed at 37°C in the carbon dioxide incubator under 5 % carbon dioxide and 100 % humidity. Liquid culture medium was Iscove's modified Dulbecco's medium (IMDM, GIBCO-BRL, U.S.A.) added with 2 % BSA, 10  $\mu$ g/ml human insulin, 200  $\mu$ g/ml transferrin, 40  $\mu$ g/ml low density lipoprotein (GIBCO-BRL, U.S.A.),  $10^{-5}$ M 2-mercaptoethanol, 50 ng/ml human SCF, 5 ng/ml human IL-3, 10 ng/ml human IL-6, 5 ng/ml human GM-CSF (Intergen Inc., U.S.A.), and 3 U/ml Epo. If necessary, HDEXIg-500ng or 50 ng/ml MIP-1  $\alpha$  (Intergen Inc., U.S.A.) was added. The medium was added 1 ml/well and half of the medium was changed three times in a week. After culturing 2, 4, 6 and 8 weeks, all cells were

collected from wells by using cell scraper in 1.5 ml micro tube. Cells were precipitated by centrifugation and resuspended in a fresh IMDM 1 ml, counted the cell counts by using hemocytometer, and in 5000 cells/ml, blood cell colony forming assay was performed.

Blood cell colony forming assay was performed using the Iscove's methylcellulose complete ready mix (Stem Cell Technologies Inc., Canada), and each 1 ml was inoculated in two plates of 35 mm dish (Falcon Inc., U.S.A.) and incubated for 2 weeks in the carbon dioxide incubator. Blood colonies were counted CFU-GM and BFU-E in the invert microscope, and total was counted as CFU-C. CFU-C counts and cell counts obtained by hemocytometer were multiplied to obtain CFU-C count/1000 cells inoculated in the liquid culture.

In Table 1, result of HDEXIg and in Table 2, result of HSEXIg are shown. Experiments were conducted at  $n = 3$ , values obtained were shown by (mean  $\pm$  SD). In the table, ND means no detection of colony.

Table 1 Colony forming cell maintenance action in the long-term liquid culture of human Delta-1 of the present invention

Week	Cytokines		
	—	MIP-1 $\alpha$	HDEXIg
0	69 $\pm$ 9	68 $\pm$ 9	68 $\pm$ 9
2	1440 $\pm$ 120	720 $\pm$ 110	1280 $\pm$ 230
4	340 $\pm$ 40	420 $\pm$ 80	410 $\pm$ 90
6	28 $\pm$ 6	96 $\pm$ 17	290 $\pm$ 60
8	ND	ND	88 $\pm$ 13

Table 2 Colony forming cell maintenance action in the long-term liquid culture of human Serrate-1 of the present invention

Week	Cytokines		
	—	MIP-1 $\alpha$	HSEXIg
0	68 $\pm$ 9	68 $\pm$ 9	68 $\pm$ 9
2	1440 $\pm$ 120	720 $\pm$ 110	1360 $\pm$ 280
4	340 $\pm$ 40	420 $\pm$ 80	560 $\pm$ 70
6	28 $\pm$ 6	96 $\pm$ 17	220 $\pm$ 50
8	ND	ND	130 $\pm$ 50

CFU-C could only be observed until 6<sup>th</sup> week of cultivation under the condition without cytokines for maintaining undifferentiated condition, and under the condition with MIP-1  $\alpha$ . It could be observed at 8<sup>th</sup> week in the presence of HDEXIg or HSEXIg. In comparison with MIP-1  $\alpha$  and HDEXIg and HSEXIg, MIP-1  $\alpha$  strongly suppressed colony formation at 2 weeks of culture, however no suppression in HDEXIg and HSEXIg were observed. In maintenance of CFU-C counts at 6 and 8 weeks of culture, HDBXlg and HSEXIg were superior.

#### Example 12

Effects of HDEXIg and HSEXIg on liquid culture of blood undifferentiated cell LTC-IC

In order to observing physiological action of HDEXIg and HSEXIg on the blood undifferentiated cells, umbilical cord blood CD34 positive cells were cultured for two weeks in the serum-free liquid medium in the presence of HDEXIg or HSEXIg and known cytokines, and numbers of LTC-IC, which was thought to be most undifferentiated blood cells at present were observed.

The umbilical cord blood monocyte CD34 positive cells, 100000 to 20000 cells, separated by a method described in Example 10 were cultured in the following medium for 2 weeks. Numbers of LTC-IC in 4 experimental

groups, which include a group before cultivation, a group of HDEXIg, a group of HSEXIg and a control group, were determined. Media used in liquid culture medium were  $\alpha$  - medium added with 2 % BSA, 10  $\mu$ g/ml human insulin, 200  $\mu$ g/ml transferrin, 40  $\mu$ g/ml low density lipoprotein, and  $10^{-5}$ M 2-mercaptoethanol, further added with 100 ng/ml human SCF, 10 ng/ml human IL-3, and 100 ng/ml human IL-6. HDEXIg or HSEXIg 1  $\mu$ g/ml were added to the above medium. In the control group, human IgG1 was added in the equal concentration.

Preparation of human bone marrow stromal cell layer used for LTC-IC, and quantitative assay of frequency of LTC-IC by a limit dilution were performed according to a method of Sutherland et al. (Blood, 74, 1563-, 1989 and Proc. Natl. Acad. Sci. USA, 87, 3584-, 1990 ).

The bone marrow mononuclear cells,  $1-2 \times 10^7$  cells, obtained in Example 10 before the separation and without the silica solution treatment, were cultured in LTC medium (MyeloCul, Stem Cell Technologies Inc., Canada) 5 ml added with hydrocortisone  $1 \mu$ M (Upjohn Japan Co., Japan ) in T-25 flask (Falcon Inc., U.S.A. ) at 37°C under 5 % carbon dioxide and 100 % humidity in the carbon dioxide incubator. Culture was conducted until the adhesive cell layers of the stromal cell formation spread more than 80 % of the bottom area of the culture. Detachment of the cell layer was performed by treating with EDTA solution (Cosmobio Co., Japan). Cells were plated in the 96 well plate (Beckton-Deckinson Inc., U.S.A.), about  $2 \times 10^4$  cells/well and re-cultivation was continued in the same medium. X-ray, 15Gy, 250 KV peak was irradiated after reconstituted stromal cell layer. Growth of stromal cells was stopped and blood cells in the stromal cells were removed by this treatment. The thus prepared stromal cells were used as stromal cell layer for the experiments.

In the assay of LTC-IC, cell counts in each group were adjusted within the ranges of 25-400 cells/well for CD34 positive cells before the cultivation, and 625-20000 cells/well for the cells after the cultivation, and cells were diluted for six step-dilution within these ranges. Each dilution step of cells was co-cultured with the above stromal

cell layer in the 96 well plate, for 16 wells/cells of one dilution step. Culture was performed in the same medium as used in stromal formation, at 37°C under 5 % carbon dioxide and 100 % humidity in the carbon dioxide gas incubator for 5 weeks. Cells in suspension and in attachment after cultivation were recovered in each well. Collected cells were transferred to the semi-solid culture medium consisting of  $\alpha$ -medium added with 0.9 % methylcellulose, 30 % fetal calf serum (FCS, ICN Biomedical Japan), 1 % BSA,  $10^{-5}$ M 2-mercaptoethanol, 100 ng/ml human SCF, 10 ng/ml human IL-3, 100 ng/ml human IL-6, 2U/ml Epo and 10 ng/ml human G-CSF. After 2 weeks of cultivation, colony forming cells were detected as the same was as described in Example 10 and 11, and numbers of well, in which colony forming cells were found, were detected. Incidence of LTC-IC was calculated according to the method of Taswell et al. (J. Immunol. 126, 1614-, 1981) based on the above data.

Graph used for calculation is shown in Fig.4. In Fig.4, calculation curves after liquid culture is shown. A vertical axis shows ratio of well for no colonies were observed, and a horizontal axis shows number of cells/well. In each experimental group, numbers of well, for which colonies were not observed, and numbers of cells were plotted, then regression curve was calculated by the least square method. Number of cells corresponding to number of 0.37 (a reciprocal of a base of natural logarithm) for which colonies did not appeared, was calculated. A reciprocal of that number of cells is a frequency of LTC-IC. Further, absolute number of LTC-IC was calculated from initial number of cells and frequency of LTC-IC.

The result indicated that 243 LTC-IC were found in 25000 cells before the liquid culture. In the control group number of cells during 2 weeks of cultivation increased to 1,510,000 cells, and LTC-IC was decreased to 49 cells. However, culturing with human Delta-1, i.e. HDEXIg or human Serrate-1, i.e. HSEXIg, numbers of cells were maintained in 1,310,000 and 1,140,000, respectively, and numbers of LTC-IC were slightly decreased to 115 and 53. Consequently, polypeptide of the present invention,

especially human Delta-1 could have an activity for maintenance of number of LTC-IC in the liquid culture.

### Example 13

#### Binding of HDEXIg and HSEXIg for blood cells

Binding of Notch ligands with various blood cells was studied using specific binding of Notch ligands to Notch receptors.

Blood cell lines tested were Jurkat (ATCC TIB-152), Namalwa (ATCC CRL-1432), HL-60 (ATCC CRL-1964), K562 (ATCC CCL-243), THO-1 (ATCC TIB-2 02), UT-7 (Komatsu et al., Cancer Res., 51, 341-348, 1991), Mo7e (Avanzi et al. Br. J. Haematol., 69, 359-, 1988) and CMK (Sato et al. Exp. Hematol., 15, 495-502, 1987). Culturing media for these cells were found in the reference or ATCC CELL LINES & HYBRIDOMAS, 8<sup>th</sup> Ed. (1994).

Cells,  $1 \times 10^6$  cells, were suspended in Hank's balanced salt solution containing 2% FCS and 10 mM Hepes. HDEXIg or HSEXIg  $1 \mu\text{g/ml}$  were added therein and allowed to stand at  $4^\circ\text{C}$  for overnight. Cells were washed twice with the Hank's solution. PE labeled sheep anti-human IgG monoclonal antibody  $1 \mu\text{g/ml}$  was added, allow to stand in ice-cooling for 30 minutes, washed twice with the Hank's solution, and suspended in the Hank's solution 1 ml. Analysis was performed using the flow cytometer (FACSCalibur). Control groups were used with human IgG1 staining in place of HDEXIg or HSEXIg staining.

Results are shown in Fig. 5. A vertical axis indicates cell counts and a horizontal axis indicates fluorescence intensity. Staining with HDEXIg or HSEXIg is shown by solid line and control, a staining with human IgG1 is shown by a broken line. In Fig. 5, the left column shows HDEXIg and the right column shows HSEXIg. As shown in Fig. 5, results indicate that Jurkat : reacted, Namalwa ; non-reacted, HL-60 : non-reacted, K562 : non-reacted, THP-1 : non-reacted, UT-7 : reacted, Mo7e : non-reacted and CMK : reacted. Since the same results in HDEXIg and HSEXIg were obtained, both recognized the identical molecule and these cells can be differentiated.

Effect of the invention

Notch ligand molecules of the present invention can be used for maintenance of undifferentiated-suppressive substances, and in the preparation of pharmaceuticals.

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